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(54) Title: DIAGNOSIS OF HYPERINSULINEMIA AND TYPE II DIABETES AND PROTECTION AGAINST SAME (I)

(57) Abstract: Human subjects who are prone to progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, may be identified by suitably screening for one or more "favorable" or "unfavorable" human marker genes, or their encoded proteins. "Favorable" genes and proteins, and antagonists of "unfavorable ones," are also useful in therapy.

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DIAGNOSIS OF HYPERINSULINEMIA AND TYPE II DIABETES AND PROTECTION AGAINST SAME (I)

This application claims the benefit under 35 USC 119(e) of U.S. Provisional Appl. 60/458,398, filed March 31, 2003, which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to various nucleic acid molecules and proteins, and their use in (1) diagnosing hyperinsulinemia and type II diabetes, or conditions associated with their development, and (2) protecting mammals (including humans) against them.

Description of the Background Art

Diabetes

Diabetes mellitus is a pleiotropic disease of great complexity. The two major types have been termed type I or insulin-dependent diabetes mellitus (IDDM) and type II or non-insulin-dependent diabetes mellitus (NIDDM. Type II diabetes is the predominant form found in the Western world; fewer than 8% of diabetic Americans have the type I disease.

Type I diabetics are often characterized by their low or absent levels of circulating endogenous insulin, i.e., hypoinsulinemia (Unger and Foster, 1998). Islet cell antibodies causing damage to the pancreas are frequently present at diagnosis. Injection of exogenous insulin is required to prevent ketosis and sustain life.

Early Type II diabetics are often characterized by hyperinsulinemia and high resistance to insulin. Late Type II diabetics may be normoinsulinemic or hypoinsulinemic. Type II diabetics are usually not insulin dependent or prone to ketosis under normal circumstances.

Type II Diabetes

Type II diabetes (formerly known as non-insulin dependent diabetes, NIDDM) is the most common form of

elevated blood glucose (hyperglycemia). Type II diabetes is a metabolic disorder that affects approximately 17 million Americans. It is estimated that another 10 million individuals are "prone" to becoming diabetic. These vulnerable individuals can become resistant to insulin, a pancreatic hormone that signals glucose (blood sugar) uptake by fat and muscle. In order to maintain normal glucose levels, the islet cells of the pancreas produce more insulin, resulting in a condition called hyperinsulinemia. When the pancreas can no longer produce enough insulin to compensate for the insulin resistance, and thereby maintain normal glucose levels, Type II diabetes (hyperglycemia) results.

Complications of diabetes (end organ damage) include retinopathy, neuropathy, and nephropathy (traditionally designated as microvascular complications) as well as atherosclerosis (a macrovascular complication).

Early stages of hyperglycemia can usually be controlled by an alteration in diet and increasing the amount of exercise, but drug treatment, including insulin, may be required. It has been shown that meticulous blood glucose control can often slow down or halt the progression of diabetic complications if caught early enough (Unger and Foster, 1998). However, tight metabolic control is extremely difficult to achieve.

Little is known about the disease progression from the normoinsulinemic state to the hyperinsulinemic state, and from the hyperinsulinemic state to the Type II diabetic state. As stated above, type II diabetes is a metabolic disorder that is characterized by insulin resistance and impaired glucose-stimulated insulin secretion. However, Type II diabetes and atherosclerotic disease are viewed as consequences of having the insulin resistance syndrome (IRS) for many years. The current theory of the pathogenesis of Type II diabetes is often referred to as the "insulin resistance/islet cell exhaustion" theory. According to this theory, a condition causing insulin resistance compels the pancreatic islet cells to hypersecrete insulin in order to

maintain glucose homeostasis. However, after many years of hypersecretion, the islet cells eventually fail and the symptoms of clinical diabetes are manifested. Therefore, this theory implies that, at some point, peripheral hyperinsulinemia will be an antecedent of Type II diabetes. Peripheral hyperinsulinemia can be viewed as the difference between what is produced by the β cell minus that which is taken up by the liver. Therefore, peripheral hyperinsulinemia can be caused by increased β cell production, decreased hepatic uptake or some combination of both. It is also important to note that it is not possible to determine the origin of insulin resistance once it is established since the onset of peripheral hyperinsulinemia leads to a condition of global insulin resistance.

Multiple environmental and genetic factors are involved in the development of insulin resistance, hyperinsulinemia and type II diabetes. An important risk factor for the development of insulin resistance, hyperinsulinemia and type II diabetes is obesity, particularly visceral obesity. The disease exists world-wide, but in developed societies, the prevalence has risen as the average age of the population increases and the average individual becomes more obese.

Obesity is a serious and growing problem in the United States. Obesity-related health risks include high blood pressure, hardening of the arteries, cardiovascular disease, and Type II diabetes (also known as non-insulin-dependent diabetes mellitus, Type II diabetes). Recent studies show that 85% of the individuals with Type II diabetes are obese.

Growth Hormone

Growth hormone has many roles, ranging from regulation of protein, fat and carbohydrate metabolism to growth promotion. GH is produced in the somatrophic cells of the anterior pituitary and exerts its effects either through the GH-induced action of IGF-I, in the case of growth promotion, or by direct interaction with the GHR on target cells including liver, muscle, adipose, and kidney cells.

Hyposecretion of GH during development leads to dwarfism, and hypersecretion before puberty leads to gigantism. In adults, hypersecretion of GH results in acromegaly, a clinical condition characterized by enlarged facial bones, hands, feet, fatigue and an increase in weight. Of those individuals with acromegaly, 25% develop type II diabetes. This may be due to insulin resistance caused by the high circulating levels of GH leading to high circulating levels of insulin (Kopchick et al., Annual Rev. Nutrition 1999. 19:437-61).

A further mode of GH action may be through the transcriptional regulation of a number of genes contributing to the physiological effects of GH.

Transgenic Mice

McGrane, et al., J. Biol. Chem. 263:11443-51 (1988) and Chen, et al., J. Biol. Chem., 269:15892-7 (1994) describe the genetic engineering of mice to express bovine growth hormone (bGH) or human growth hormone (hGH), respectively. These mice exhibited an enhanced growth phenotype. They also developed kidney lesions similar to those seen in diabetic glomerulosclerosis, see Yang, et al., Lab. Invest., 68:62-70 (1993). Ogueta, et al., J. Endocrinol., 165: 321-8 (2000) reported that transgenic mice expressing bovine GH develop arthritic disorder and self-antibodies.

Growth hormone genes and the proteins encoded by them can be converted into growth hormone antagonists by mutation, see Kopchick USP 5,350,836. Transgenic mice have been made that express the GH antagonists bGH-G119R or hGH G120R, and which exhibit a dwarf phenotype. Chen, et al., J. Biol. Chem., 263:15892-7 (1994); Chen, et al., Mol. Endocrinol, 5:1845-52 (1991); Chen, et al., Proc. Nat. Acad. Sci. USA 87:5061-5 (1990). These mice did not develop kidney lesions. See Yang (1993), supra.

Chen, et al., Endocrinol, 136:660-7 (1995) compared the effect of streptozotocin treatment in normal nontransgenic mice, and in mice transgenic for (1) a GH receptor

antagonist, the G119R mutant of bovine growth hormone or (2) the E117L-mutant of bGH. (According to Chen's ref. 24, these large GH transgenic streptozotocin-treated mice constitute an animal model for diabetes.) Glomerulosclerosis was seen in diabetic (STZ-treated) nontransgenic mice and in diabetic bGH-E117L mice, but not in diabetic bGH-G119R (GH antagonist) mice.

Two of the proteins which mediate growth hormone activity are the growth hormone receptor and the growth hormone binding protein, encoded by the same gene in mice(GHR/BP). It is possible to genetically engineer mice so that the gene encoding these proteins is disrupted ("knocked-out"; inactivated), see Zhou, et al., Proc. Nat. Acad. Sci. (USA), 94:13215-20 (1997). Zhou, et al. inactivated the GHR/BP gene by replacing the 3' portion of exon 4 (which encodes a portion of the GH binding domains) and the 5' region of intron 4 with a neomycin gene cassette. The modified gene was introduced into the target mice by homologous recombination. Like mice expressing a GH antagonist, homozygous GHR/BP-KO mice exhibit a dwarf phenotype. GHR/BP-KO mice, made diabetic by streptozotocin treatment, are protected from the development of diabetes-associated nephropathy. Bellush, et al., Endocrinol., 141:163-8 (2000).

Differential/Subtractive Hybridization

Zhang, et al., Kidney International, 56:549-558 (1999) identified genes up-regulated in 5/6 nephrectomized (subtotal renal ablation) mouse kidney by a PCR-based subtraction method. Ten known and nine novel genes were identified. The ultimate goal was to identify genes involved in glomerular hyperfiltration and hypertrophy.

Melia, et al., Endocrinol., 139:688-95 (1998) applied subtractive hybridization methods for the identification of androgen-regulated genes in mouse kidney. The treatment mice were dosed with dihydrotestosterone, an androgen.

Kidney androgen-regulated protein gene was used as a positive control, as it is known to be up-regulated by DHT.

See also Holland, et al., Abstract 607, "Identification of Genes Possibly Involved in Nephropathy of Bovine Growth Hormone Transgenic Mice" (Endocrine Society Meeting, June 22, 2000) and Coschigano, et al., Abstract 333, "Identification of Genes Potentially Involved in Kidney Protection During Diabetes" (Endocrine Society Meeting, June 22, 2000).

The following differential hybridization articles may also be of interest:

Wada, et al., "Gene expression profile in streptozotocin-induced diabetic mice kidneys undergoing glomerulosclerosis", Kidney Int, 59:1363-73 (2001);

Song, et al., "Cloning of a novel gene in the human kidney homologous to rat muncl3S: its potential role in diabetic nephropathy", Kidney Int., 53:1689-95 (1998);

Page, et al., "Isolation of diabetes-associated kidney genes using differential display", Biochem. Biophys. Res. Comm., 232:49-53 (1997).

Peradi, "Subtractive hybridization claims: An efficient technique to detect overexpressed mRNAs in diabetic nephropathy," Kidney Int. 53:926-31 (1998).

Condorelli, EMBO J., 17:3858-66 (1998).

See also WO00/66784 (differential hybridization screening for brown adipose tissue); PCT/US00/12366, filed May 5, 2000 (differential hybridization screening for liver).

Identification of genes involved in hyperinsulinemia and type II diabetes

High-fat diets have been shown to induce both obesity and Type II diabetes in laboratory animals (Surwit et al., 1988). Surwit and colleagues demonstrated that male C57BL/6J mice are extremely sensitive to the diabetogenic effects of a high-fat diet when initiated at weaning. At six months of age, high-fat fed animals had significantly elevated fasting blood-glucose and insulin levels and also

demonstrated a decrease in insulin sensitivity (Surwit et al., 1995). Ahren and colleagues (Ahren et al., 1997) reported evidence of insulin resistance as well as diminished glucose-stimulated insulin release, after feeding with a high-fat diet for 12 weeks. These mice also showed elevated levels of total cholesterol, triglycerides, and free fatty acids, another hallmark of Type II diabetes.

Our attention recently has focused on the generation of liver mRNA expression profiles and the identification of genes involved in the genesis of the obesity-induced hyperinsulinemia and type-II diabetes. To date, no one has attempted to study the actual progression from the normal condition to that of hyperinsulinemia or from hyperinsulinemia to Type II diabetes in an attempt to identify genes that are up-regulated or down-regulated as the disease progresses.

In previous studies aimed at identifying genes involved in diabetes-induced glomerulosclerosis, differential display and traditional subtractive hybridization techniques were used (Page et al., 1997; Condorelli et al., 1998; Peraldi et al., 1998; Song et al., 1998; Imagawa et al., 1999). While effective for the identification of a few genes (e.g. hmunc13, PED/PEA-15, lactate dehydrogenase, amiloride sensitive sodium channel, ubiquitin-like protein, mdr 1, and a-amyloid protein precursor as well as a few novel genes), these techniques can be quite labor intensive. The PCR-based method of subtractive hybridization requires less starting material, and allows the simultaneous isolation of all differentially expressed cDNAs into two groups (up-regulated and down-regulated).

SUMMARY OF THE INVENTION

We have studied changes in gene expression patterns in the insulin target tissue, liver, during this progression. Our underlying hypothesis is that this insulin sensitive tissue sends and receives signaling molecules and that these signaling molecules change during the progression of the disease. We have excised liver tissue from mice and used it to identify genes whose expression is either up- or down regulated during this progression. By identifying signaling molecules involved in the progression from normal to hyperinsulinemic, and from hyperinsulinemic to Type II diabetes, we hope to be able to intervene in the disease process.

Differential (subtractive) hybridization techniques have been used to identify mouse genes that are differentially expressed in mice, depending upon their development of hyperinsulinemia or type II diabetes. Since liver is a target for the action of insulin, and is the only organ in the body that can synthesize and secrete glucose, and is thereby affected as hyperinsulinemia progresses toward Type II diabetes, we concentrated our efforts on this tissue.

After identifying related human genes and proteins, one may formulate agents useful in screening humans at risk for progression toward hyperinsulinemia or toward type II diabetes.

Since the progression is from normal to hyperinsulinemic, and thence from hyperinsulinemic to type II diabetic, one may define mammalian subjects as being more favored or less favored, with normal subjects being more favored than hyperinsulinemic subjects, and hyperinsulinemic subjects being more favored than type II diabetic subjects. The subjects' state may then be correlated with their gene expression activity.

Thus, "favorable" human genes/proteins are defined as those corresponding to mouse cDNAs which were less strongly expressed in mouse hyperinsulinemic liver than in control liver, or less strongly expressed in mouse type II diabetic

liver than in control or hyperinsulinemic liver. (The control liver is the liver of a mouse which is normal vis-a-vis fasting insulin and fasting glucose levels . The term "normal", as used herein, means normal relative to those parameters, and does not necessitate that the mouse be normal in every respect.)

Likewise, one may define "unfavorable" human genes/proteins as those corresponding to mouse cDNAs which were more strongly expressed in mouse hyperinsulinemic liver than in control liver, or more strongly expressed in mouse type II diabetic liver than in control or hyperinsulinemic liver.

As used herein, the term "corresponding" does not mean identical, but rather implies the existence of a statistically significant sequence similarity, such as one sufficient to qualify the human protein or gene as a homologus protein or DNA as defined below. The greater the degree of relationship as thus defined (i.e., by the statistical significance of each alignment used to connect the mouse cDNA to the human protein or gene, measured by an E value), the more close the correspondence. The connection may be direct (mouse cDNA to human protein) or indirect (e.g., mouse cDNA to mouse gene, mouse gene to human protein). In general, the human genes which most closely correspond, directly or indirectly, to the mouse cDNA are preferred, such as the one(s) with the highest, top two highest, top three highest, top four highest, top five highest, and top ten highest E values for the final alignment in the connection process. The human genes/proteins deemed to correspond to our mouse cDNA clones are identified in the Master Tables.

A human gene/protein corresponding to a mouse cDNA which was more strongly expressed in hyperinsulinemic liver than in either normal or type II diabetic liver (i.e., C<IR, IR>D) will be deemed both "unfavorable", by virtue of the control:hyperinsulinemic comparison, and "favorable", by

virtue of the hyperinsulinemic:diabetic comparison. This is one of several possible "mixed" expression patterns.

Thus, we can subdivide the "favorables" into wholly and partially favorables. Likewise, we can subdivide the unfavorables into wholly and partially unfavorables. The genes/proteins with "mixed" expression patterns are, by definition, both partially favorable and partially unfavorable. In general, use of the wholly favorable or wholly unfavorable genes/proteins is preferred to use of the partially favorable or partially unfavorable ones.

Agents which bind the "favorable" and "unfavorable" nucleic acids (e.g., the agent is a substantially complementary nucleic acid hybridization probe), or the corresponding proteins (e.g., the agent is an antibody vs. the protein) may be used to evaluate whether a human subject is at increased or decreased risk for progression toward type II diabetes. A subject with one or more elevated "unfavorable" and/or one or more depressed "favorable" genes/proteins is at increased risk, and one with one or more elevated "favorable" and/or one or more depressed "unfavorable" genes/proteins is at decreased risk. One may further take into account whether the subject is normoinsulinemic or hyperinsulinemic at the time of the assay. If the subject is non-diabetic and normoinsulinemic, we are especially interested in the "favorable" and "unfavorable" genes/proteins corresponding to mouse cDNAs differentially expressed in hyperinsulinemic vs. normal livers. If the subject is non-diabetic, but hyperinsulinemic, we are especially interested in the "favorable" and "unfavorable" genes/proteins corresponding to mouse cDNAs differentially expressed in type II diabetic vs. hyperinsulinemic livers.

The assay may be used as a preliminary screening assay to select subjects for further analysis, or as a formal diagnostic assay.

The identification of the related genes and proteins may also be useful in protecting humans against these disorders.

Thus, Applicants contemplate:

(1) use of the "favorable" mouse DNAs of the Master Table (below) to isolate or identify related human DNAs;

(2) use of human DNAs, related to favorable mouse DNAs, to express the corresponding human proteins;

(3) use of the corresponding human proteins (and mouse proteins, if the sequence is sufficiently complete to be biologically active, and is active in humans), to protect against the disorder(s);

(4) use of the corresponding mouse or human proteins, or nucleic acid probes, in diagnostic agents, in assays to measure progression toward hyperinsulinemia or type II diabetes, or protection against the disorder(s), or to estimate related end organ damage such as kidney damage; and

(5) use of the corresponding human or mouse genes therapeutically in gene therapy, to protect against the disorder(s).

Moreover Applicants contemplate:

(1) use of the "unfavorable" mouse DNAs of the Master Table to isolate or identify related human DNAs;

(2) use of the complement to the "unfavorable" mouse DNAs or related human DNAs, as antisense molecules to inhibit expression of the related human DNAs;

(3) use of the mouse or human DNAs to express the corresponding mouse or human proteins;

(4) use of the corresponding mouse or human proteins, or nucleic acid probes, in diagnostic agents;

(5) use of the corresponding mouse or human proteins in assays to determine whether a substance binds to (and hence may neutralize) the protein; and

(6) use of the neutralizing substance to protect against the disorder(s).

The related human DNAs may be identified by comparing the mouse sequence (or its AA translation product) to known human DNAs (and their AA translation products). If this is

unsuccessful, human cDNA or genomic DNA libraries may be screened using the mouse DNA as a probe.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

Subjects

A mouse is considered to be a diabetic subject if, regardless of its fasting plasma insulin level, it has a fasting plasma glucose level of at least 190 mg/dL. A mouse is considered to be a hyperinsulinemic subject if its fasting plasma insulin level is at least 0.67 ng/mL and it does not qualify as a diabetic subject. A mouse is considered to be "normal" if it is neither diabetic nor hyperinsulinemic. Thus, normality is defined in a very limited manner.

A mouse is considered "obese" if its weight is at least 15% in excess of the mean weight for mice of its age and sex. A mouse which does not satisfy this standard may be characterized as "non-obese", the term "normal" being reserved for use in reference to glucose and insulin levels as previously described.

A human is considered a diabetic subject if, regardless of his or her fasting plasma insulin level, the fasting plasma glucose level is at least 126 mg/dL. A human is considered a hyperinsulinemic subject if the fasting plasma insulin level is more than 26 micro International Units/mL (it is believed that this is equivalent to 1.08 ng/mL), and does not qualify as a diabetic subject. A human is considered to be "normal" if it is neither diabetic nor hyperinsulinemic. Thus, normality is defined in a very limited manner.

A human is considered "obese" if the body mass index (BMI) (weight divided by height squared) is at least 30 kg/m^2. A human who does not satisfy this standard may be characterized as "non-obese", the term "normal" being reserved for use in reference to glucose and insulin levels as previously described.

A human is considered overweight if the BMI is at least 25 kg/m^2. Thus, we define overweight to include obese

individuals, consistent with the recommendations of the National Institute of Diabetes and Digestive and Kidney Diseases(NIDDK). A human who does not satisfy this standard may be characterized as "non-overweight."

According to the Report of the Expert Committe on the Diagnosis and Classification of Diabetes Mellitus, Diabetes Care 20: 1183-97 (1997), the following are risk factors for diabetes type II:

older (e.g., at least 45; see below)

excessive weight (see below)

first-degree relative with diabetes mellitus

member of high risk ethnic group (black, Hispanic, Native American, Asian)

history of gestational diabetes mellitus or delivering a baby weighing more than 9 pounds (4.032 kg)

hypertensive (>140/90 mm Hg)

HDL cholesterol level >35 mg/dL (0.90 mmol/L)

triglyceride level >=250 mg/dL (2.83 mmol/L)

Hence, in a preferred embodiment, the diagnostic and protective methods of the present invention are applied to human subjects exhibiting one or more of the aforementioned risk factors. Likewise, in a preferred embodiment, they are applied to human subjects who, while not diabetic, exhibit impaired glucose homeostasis (110 to <126 mg/dL).

The risk of diabetes increases with age. Hence, in successive preferred embodiments, the age of the subjects is

at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, and at least 75.

With regard to excessive weight, NIDDK says that "The relative risk of diabetes increases by approximately 25 percent for each additional unit of BMI over 22." Hence, in successive preferred embodiments, the BMIs of the human subjects is at least 23, at least 24, at least 25 (i.e., overweight by our criterion), at least 26, at least 27, at least 28, at least 29, at least 30 (i.e., obese), at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, or over 40.

Identified Differentially Expressed cDNAs

We have performed differential expression studies comparing genes expressed in normal (control, C), hyperinsulinemic (HI) and type II diabetic (D) livers of mice.

Summary of Expression Patterns:

Clone	C:HI	C:D	HI:D
Z41	F	F	No Change
Z74	F	F	F
Y92	F	No Change	U
Z19	F	F	No Change
A17	U	U	F
A53	U	No Change	F
A104	U	U	No Change
B8	U	No Change	F
B39	U	U	No Change
Y68	U	U	No Change
Y89	U	U	No Change
Y91	U	U	U

Mixed genes/proteins are those exhibiting a combination of favorable and unfavorable behavior. They are considered to be both favorable and unfavorable for the purpose of the claims. A mixed gene/protein can be used as would a favorable gene/protein if its favorable behavior outweighs the unfavorable. It can be used as would an unfavorable gene/protein if its unfavorable behavior outweighs the favorable. Preferably, they are used in conjunction with other agents that affect their balance of favorable and unfavorable behavior. Use of mixed genes/proteins is, in general, less desirable than use of purely favorable or purely unfavorable genes/proteins.

Genes/Proteins of Interest

Favorable genes/proteins are those corresponding to cDNAs less strongly expressed in type II diabetic or hyperinsulinemic liver than in normal liver, or less strongly expressed in type II diabetic liver than in hyperinsulinemic liver Unfavorable genes/proteins are those corresponding to cDNAs more strongly expressed in hyperinsulinemic or type II diabetic liver as compared to normal liver, or in type II diabetic liver as compared to hyperinsulinemic liver.

For each of the differentially expressed cDNAs, corresponding mouse and human proteins have been identified, as set forth in Master Table 1. More than one human protein may be identified as corresponding to a particular mouse clone. In addition, we have considered whether these cDNAs may correspond to particular classes or subclasses of human proteins, as set forth in Master Table 2.

Direct and Indirect Utility of Identified Nucleic Acid Sequences and Related Molecules

The cDNAs of the disclosed clones may be used directly. For diagnostic or screening purposes, they (or specific binding fragments thereof) may be labeled and used as hybridization probes. For therapeutic purposes, they (or specific binding fragments thereof) may be used as antisense

reagents to inhibit the expression of the corresponding gene, or of a sufficiently homologous gene of another species.

If the cDNA appears to be a full-length cDNA, that is, that it encodes an entire, functional protein, then it may be used in the expression of that protein. Such expression may be in cell culture, with the protein subsequently isolated and administered exogenously to subjects who would benefit therefrom, or in vivo, i.e., administration by gene therapy. Naturally, any DNA encoding the same protein, or a fragment or a mutant protein which retains the desired activity, may be used for the same purpose. The encoded protein of course has utility therapeutically and, in labeled or immobilized form, diagnostically.

The cDNAs of the disclosed clones may also be used indirectly, that is, to identify other useful DNAs, proteins, or other molecules. We have attempted to determine whether the cDNAs disclosed herein have significant similarity to any known DNA, and whether, in any of the six possible combinations of reference frame and strand, they encode a protein similar to a known protein. If so, then it follows that the known protein, and DNAs encoding that protein, may be used in a similar manner. In addition, if the known protein is known to have additional homologues, then those homologous proteins, and DNAs encoding them, may be used in a similar manner.

There thus are several ways that a human protein homologue of interest can be identified by database searching, including but not limited to:

1) a DNA->DNA (BlastN) search for database DNAs closely related to the mouse cDNA clone identifies a particular mouse (or other nonhuman, e.g., rat) gene, and that nonhuman gene encodes a protein for which there is a known human protein homologue;

2) a DNA->Protein (BlastX) search for database proteins

closely related to the translated DNA of the mouse cDNA clone identifies a particular mouse (or other nonhuman) protein, and that nonhuman protein has a known human protein homologue;

3) a DNA->DNA (BlastN) search of the database for human DNAs closely related to the mouse cDNA clone identifies a particular human DNA as a homologue of the mouse cDNA, and the corresponding human protein is known (e.g., by translation of the human DNA); and

4) a DNA->Protein (BlastX) search of the database for human proteins closely related to the translated DNA of the mouse cDNA clone identifies a particular human protein as a homologue of the corresponding mouse protein.

Thus, if we have identified a mouse cDNA, and it encodes a mouse protein which appears similar to a human protein, then that human protein may be used (especially in humans) for purposes analogous to the proposed use of the mouse protein in mice. Moreover, a specific binding fragment of an appropriate strand of the corresponding human gene or cDNA could be labeled and used as a hybridization probe (especially against samples of human mRNA or cDNA).

In determining whether the disclosed cDNAs have significant similarities to known DNAs (and their translated AA sequences to known proteins), one would generally use the disclosed cDNA as a query sequence in a search of a sequence database. The results of several such searches are set forth in the Examples. Such results are dependent, to some degree, on the search parameters. Preferred parameters are set forth in Example 1. The results are also dependent on the content of the database. While the raw similarity score of a particular target (database) sequence will not vary with content (as long as it remains in the database), its informational value (in bits), expected value, and relative ranking can change. Generally speaking, the changes are small.

It is possible to use the sequence of the entire cDNA insert to query the database. However, the error rate increases as a sequencing run progresses. Hence, it may be beneficial to search the database using a truncated (presumably more accurate) sequence, especially if the insert is quite long.

It will be appreciated that the nucleic acid and protein databases keep growing. Hence a later search may identify high scoring target sequences which were not uncovered by an earlier search because the target sequences were not previously part of a database.

Hence, in a preferred embodiment, the cognate DNAs and proteins include not only those set forth in the examples, but those which would have been highly ranked (top ten, more preferably top three, even more preferably top two, most preferably the top one) in a search run with the same parameters on the date of filing of this application.

If the cDNA appears to be a partial cDNA, it may be used as a hybridization probe to isolate the full-length cDNA. If the partial cDNA encodes a biologically functional fragment of the cognate protein, it may be used in a manner similar to the full length cDNA, i.e., to produce the functional fragment.

If we have indicated that an antagonist of a protein or other molecule is useful, then such an antagonist may be obtained by preparing a combinatorial library, as described below, of potential antagonists, and screening the library members for binding to the protein or other molecule in question. The binding members may then be further screened for the ability to antagonize the biological activity of the target. The antagonists may be used therapeutically, or, in suitably labeled or immobilized form, diagnostically.

If the cDNA is related to a known protein, then substances known to interact with that protein (e.g., agonists, antagonists, substrates, receptors, second messengers, regulators, and so forth), and binding molecules

which bind them, are also of utility. Such binding molecules can likewise be identified by screening a combinatorial library.

Isolation of Full Length cDNAs Using Partial cDNAs as probes

If it is determined that a cDNA of the present invention is a partial cDNA, and the cognate full length cDNA is not listed in a sequence database, the available cDNA may be used as a hybridization probe to isolate the full-length cDNA from a suitable cDNA library.

Stringent hybridization conditions are appropriate, that is, conditions in which the hybridization temperature is 5-10 deg. C. below the Tm of the cDNA as a perfect duplex.

Identification and Isolation of Homologous Genes/cDNAs Using a cDNA Probe

It may be that the sequence databases available do not include the sequence of any homologous gene, or at least of the homologous gene for a species of interest. However, given the cDNAs set forth above, one may readily obtain the homologous gene.

The possession of one cDNA (the "starting DNA") greatly facilitates the isolation of homologous genes/cDNAs. If the clone in question only features a partial cDNA, this partial cDNA may first be used as a probe to isolate the corresponding full length cDNA for the same species, and that the latter may be used as the starting DNA in the search for homologous genes.

The starting DNA, or a fragment thereof, is used as a hybridization probe to screen a cDNA or genomic DNA library for clones containing inserts which encode either the entire homologous protein, or a recognizable fragment thereof. The minimum length of the hybridization probe is dictated by the need for specificity. If the size of the library in bases is L, and the GC content is 50%, then the probe should have a length of at least l, where $L = 4^l$. This will yield, on average, a single perfect match in random DNA of L bases.

The human cDNA library is about 10^8 bases and the human genomic DNA library is about 10^{10} bases.

The library is preferably derived from an organism which is known, on biochemical evidence, to produce a homologous protein, and more preferably from the genomic DNA or mRNA of cells of that organism which are likely to be relatively high producers of that protein. A cDNA library (which is derived from an mRNA library) is especially preferred.

If the organism in question is known to have substantially different codon preferences from that of the organism whose relevant cDNA or genomic DNA is known, a synthetic hybridization probe may be used which encodes the same amino acid sequence but whose codon utilization is more similar to that of the DNA of the target organism. Alternatively, the synthetic probe may employ inosine as a substitute for those bases which are most likely to be divergent, or the probe may be a mixed probe which mixes the codons for the source DNA with the preferred codons (encoding the same amino acid) for the target organism.

By routine methods, the Tm of a perfect duplex of starting DNA is determined. One may then select a hybridization temperature which is sufficiently lower than the perfect duplex Tm to allow hybridization of the starting DNA (or other probe) to a target DNA which is divergent from the starting DNA. A 1% sequence divergence typically lowers the Tm of a duplex by 1-2°C, and the DNAs encoding homologous proteins of different species typically have sequence identities of around 50-80%. Preferably, the library is screened under conditions where the temperature is at least 20°C., more preferably at least 50°C., below the perfect duplex Tm. Since salt reduces the Tm, one ordinarily would carry out the search for DNAs encoding highly homologous proteins under relatively low salt hybridization conditions, e.g., <1M NaCl. The higher the salt concentration, and/or the lower the temperature, the greater the sequence divergence which is tolerated.

For the use of probes to identify homologous genes in

other species, see, e.g., Schwinn, et al., J. Biol. Chem., 265:8183-89 (1990) (hamster 67-bp cDNA probe vs. human leukocyte genomic library; human 0.32kb DNA probe vs. bovine brain cDNA library, both with hybridization at 42°C in 6xSSC); Jenkins et al., J. Biol. Chem., 265:19624-31 (1990) (Chicken 770-bp cDNA probe vs. human genomic libraries; hybridization at 40°C in 50% formamide and 5xSSC); Murata et al., J. Exp. Med., 175:341-51 (1992) (1.2-kb mouse cDNA probe v. human eosinophil cDNA library; hybridization at 65°C in 6xSSC); Guyer et al., J. Biol. Chem., 265:17307-17 (1990) (2.95-kb human genomic DNA probe vs. porcine genomic DNA library; hybridization at 42°C in 5xSSC). The conditions set forth in these articles may each be considered suitable for the purpose of isolating homologous genes.

Homologous Proteins and DNAs

A human protein can be said to be identifiable as homologous to a mouse cDNA clone if

(1) it can be aligned directly to the mouse cDNA clone by BlastX. and/or

(2) it can be aligned to a human gene by BlastX, whose genomic DNA (gDNA) or cDNA (DNA complementary to messenger RNA) in turn can be aligned to the mouse cDNA clone by BlastN, and/or

(3) it can be aligned to a mouse gene by BlastX, whose gDNA or cDNA in turn can be aligned to the mouse cDNA clone by BlastN, and/or

(4) it can be aligned to a mouse protein by BlastP, which in turn can be aligned to the mouse cDNA clone by BlastX, and/or

(5) it can be aligned to a mouse protein by BlastP, which in

turn can be aligned to a mouse gene by BlastX, whose gDNA or cDNA can in turn be aligned to the mouse cDNA clone by BlastN;

where any alignment by BlastN, BlastP, or BlastX is in accordance with the default parameters set forth below, and the expected value (E) of each alignment (the probability that such an alignment would have occurred by chance alone) is less than e-10.

A human gene is homologous to a mouse cDNA clone if it encodes a homologous human protein as defined above, or if it can be aligned either directly to the mouse cDNA clone, or indirectly through a mouse gene which can be aligned to said clone, according to the conditions set forth above. Preferably, two, three, four or all five of conditions (1)-(5) are satisfied.

Preferably, for each of conditions (1)-(5), for at least the final alignment (i.e., vs. the human protein), the E value is less than e-15, more preferably less than e-20, still more preferably less than e-40, further more preferably less than e-50, even more preferably less than e-60, considerably more preferably less than e-80, and most preferably less than e-100. More preferably, for those conditions in which the mouse cDNA clone is indirectly connected to the human protein by virtue of two or more successive alignments, the E value is so limited for all of said alignments in the connecting chain.

BlastN and BlastX report very low expected values as "0.0". This does not truly mean that the expected value is exactly zero (since any alignment could occur by chance), but merely that it is so infinitesimal that it is not reported. The documentation does not state the cutoff value, but alignments with explicit E values as low as e-178 (624 bits) have been reported as nonzero values, while a score of 636 bits was reported as "0.0".

Functionally homologous human proteins are also of

interest. A human protein may be said to be functionally homologous to the mouse cDNA clone if (1) there is a mouse protein which is encoded by a mouse gene whose cDNA can be aligned to the mouse cDNA clone, using BlastX with the default parameters set forth below, and the E value of the alignment is less than e-50, and (2) the human protein has at least one biological activity in common with the mouse protein.

The human proteins of interest also include those that are substantially and/or conservatively identical (as defined below) to the homologous and/or functionally homologous human proteins defined above.

Relevance of Favorable and Unfavorable Genes

If a gene is down-regulated in more favored mammals, or up-regulated in less favored mammals, (i.e., an "unfavorable gene") then several utilities are apparent.

First, the complementary strand of the gene, or a portion thereof, may be used in labeled form as a hybridization probe to detect messenger RNA and thereby monitor the level of expression of the gene in a subject. Elevated levels are indicative of progression, or propensity to progression, to a less favored state, and clinicians may take appropriate preventative, curative or ameliorative action.

Secondly, the messenger RNA product (or equivalent cDNA), the protein product, or a binding molecule specific for that product (e.g., an antibody which binds the product), or a downstream product which mediates the activity (e.g., a signaling intermediate) or a binding molecule (e.g., an antibody) therefor, may be used, preferably in labeled or immobilized form, as an assay reagent in an assay for said nucleic acid product, protein product, or downstream product (e.g., a signaling intermediate). Again, elevated levels are indicative of a present or future problem.

Thirdly, an agent which down-regulates expression of

the gene may be used to reduce levels of the corresponding protein and thereby inhibit further damage to the kidney. This agent could inhibit transcription of the gene in the subject, or translation of the corresponding messenger RNA. Possible inhibitors of transcription and translation include antisense molecules and repressor molecules. The agent could also inhibit a post-translational modification (e.g., glycosylation, phosphorylation, cleavage, GPI attachment) required for activity, or post-translationally modify the protein so as to inactivate it. Or it could be an agent which down- or up-regulated a positive or negative regulatory gene, respectively.

Fourthly, an agent which is an antagonist of the messenger RNA product or protein product of the gene, or of a downstream product through which its activity is manifested (e.g., a signaling intermediate), may be used to inhibit its activity. This antagonist could be an antibody.

Fifthly, an agent which degrades, or abets the degradation of, that messenger RNA, its protein product or a downstream product which mediates its activity (e.g., a signaling intermediate), may be used to curb the effective period of activity of the protein.

If a gene is up-regulated in more favored mammals, or down-regulated in less favored animals then the utilities are converse to those stated above.

First, the complementary strand of the gene, or a portion thereof, may be used in labeled form as a hybridization probe to detect messenger RNA and thereby monitor the level of expression of the gene in a subject. Depressed levels are indicative of damage, or possibly of a propensity to damage, and clinicians may take appropriate preventative, curative or ameliorative action.

Secondly, the messenger RNA product, the equivalent cDNA, protein product, or a binding molecule specific for those products, or a downstream product, or a signaling intermediate, or a binding molecule therefor, may be used, preferably in labeled or immobilized form, as an assay

reagent in an assay for said protein product or downstream product. Again, depressed levels are indicative of a present or future problem.

Thirdly, an agent which up-regulates expression of the gene may be used to increase levels of the corresponding protein and thereby inhibit further progression to a less favored state. By way of example, it could be a vector which carries a copy of the gene, but which expresses the gene at higher levels than does the endogenous expression system. Or it could be an agent which up- or down-regulates a positive or negative regulatory gene.

Fourthly, an agent which is an agonist of the protein product of the gene, or of a downstream product through which its activity (of inhibition of progression to a less favored state) is manifested, or of a signaling intermediate may be used to foster its activity.

Fifthly, an agent which inhibits the degradation of that protein product or of a downstream product or of a signaling intermediate may be used to increase the effective period of activity of the protein.

Mutant Proteins

The present invention also contemplates mutant proteins (peptides) which are substantially identical (as defined below) to the parental protein (peptide). In general, the fewer the mutations, the more likely the mutant protein is to retain the activity of the parental protein. The effect of mutations is usually (but not always) additive. Certain individual mutations are more likely to be tolerated than others.

A protein is more likely to tolerate a mutation which

(a) is a substitution rather than an insertion or deletion;

(b) is an insertion or deletion at the terminus, rather than internally, or, if internal, is at a domain boundary, or a loop or turn, rather than in an alpha helix or beta strand;

(c) affects a surface residue rather than an interior residue;

(d) affects a part of the molecule distal to the binding site;

(e) is a substitution of one amino acid for another of similar size, charge, and/or hydrophobicity, and does not destroy a disulfide bond or other crosslink; and

(f) is at a site which is subject to substantial variation among a family of homologous proteins to which the protein of interest belongs.

These considerations can be used to design functional mutants.

Surface vs. Interior Residues

Charged amino acid residues almost always lie on the surface of the protein. For uncharged residues, there is less certainty, but in general, hydrophilic residues are partitioned to the surface and hydrophobic residues to the interior. Of course, for a membrane protein, the membrane-spanning segments are likely to be rich in hydrophobic residues.

Surface residues may be identified experimentally by various labeling techniques, or by 3-D structure mapping techniques like X-ray diffraction and NMR. A 3-D model of a homologous protein can be helpful.

Binding Site Residues

Residues forming the binding site may be identified by (1) comparing the effects of labeling the surface residues before and after complexing the protein to its target, (2) labeling the binding site directly with affinity ligands, (3) fragmenting the protein and testing the fragments for binding activity, and (4) systematic mutagenesis (e.g., alanine-scanning mutagenesis) to determine which mutants destroy binding. If the binding site of a homologous protein is known, the binding site may be postulated by analogy.

Protein libraries may be constructed and screened that a large family (e.g., 10^8) of related mutants may be evaluated simultaneously.

Hence, the mutations are preferably conservative modifications as defined below.

"Substantially Identical"

A mutant protein (peptide) is substantially identical to a reference protein (peptide) if (a) it has at least 10% of a specific binding activity or a non-nutritional biological activity of the reference protein, and (b) is at least 50% identical in amino acid sequence to the reference protein (peptide). It is "substantially structurally identical" if condition (b) applies, regardless of (a).

Percentage amino acid identity is determined by aligning the mutant and reference sequences according to a rigorous dynamic programming algorithm which globally aligns their sequences to maximize their similarity, the similarity being scored as the sum of scores for each aligned pair according to an unbiased PAM250 matrix, and a penalty for each internal gap of -12 for the first null of the gap and -4 for each additional null of the same gap. The percentage identity is the number of matches expressed as a percentage of the adjusted (i.e., counting inserted nulls) length of the reference sequence.

A mutant DNA sequence is substantially identical to a reference DNA sequence if they are structural sequences, and encoding mutant and reference proteins which are substantially identical as described above.

If instead they are regulatory sequences, they are substantially identical if the mutant sequence has at least 10% of the regulatory activity of the reference sequence, and is at least 50% identical in nucleotide sequence to the reference sequence. Percentage identity is determined as for proteins except that matches are scored +5, mismatches -4, the gap open penalty is -12, and the gap extension penalty (per additional null) is -4.

Preferably, sequence which are substantially identical

exceed the minimum identity of 50% e.g., are 51%, 66%, 75%, 80%, 85%, 90%, 95% or 99% identical in sequence.

DNA sequences may also be considered "substantially identical" if they hybridize to each other under stringent conditions, i.e., conditions at which the Tm of the heteroduplex of the one strand of the mutant DNA and the more complementary strand of the reference DNA is not in excess of 10°C. less than the Tm of the reference DNA homoduplex. Typically this will correspond to a percentage identity of 85-90%.

"Conservative Modifications"

"Conservative modifications" are defined as

(a) conservative substitutions of amino acids as hereafter defined; or

(b) single or multiple insertions (extension) or deletions (truncation) of amino acids at the termini.

Conservative modifications are preferred to other modifications. Conservative substitutions are preferred to other conservative modifications.

"Semi-Conservative Modifications" are modifications which are not conservative, but which are (a) semi-conservative substitutions as hereafter defined; or (b) single or multiple insertions or deletions internally, but at interdomain boundaries, in loops or in other segments of relatively high mobility. Semi-conservative modifications are preferred to nonconservative modifications. Semi-conservative substitutions are preferred to other semi-conservative modifications.

Non-conservative substitutions are preferred to other non-conservative modifications.

The term "conservative" is used here in an a priori sense, i.e., modifications which would be expected to preserve 3D structure and activity, based on analysis of the naturally occurring families of homologous proteins and of past experience with the effects of deliberate mutagenesis, rather than post facto, a modification already known to

conserve activity. Of course, a modification which is conservative a priori may, and usually is, also conservative post facto.

Preferably, except at the termini, no more than about five amino acids are inserted or deleted at a particular locus, and the modifications are outside regions known to contain binding sites important to activity.

Preferably, insertions or deletions are limited to the termini.

A conservative substitution is a substitution of one amino acid for another of the same exchange group, the exchange groups being defined as follows

I Gly, Pro, Ser, Ala (Cys) (and any nonbiogenic, neutral amino acid with a hydrophobicity not exceeding that of the aforementioned a.a.'s)

II Arg, Lys, His (and any nonbiogenic, positively-charged amino acids)

III Asp, Glu, Asn, Gln (and any nonbiogenic negatively-charged amino acids)

IV Leu, Ile, Met, Val (Cys) (and any nonbiogenic, aliphatic, neutral amino acid with a hydrophobicity too high for I above)

V Phe, Trp, Tyr (and any nonbiogenic, aromatic neutral amino acid with a hydrophobicity too high for I above).

Note that Cys belongs to both I and IV.

Residues Pro, Gly and Cys have special conformational roles. Cys participates in formation of disulfide bonds. Gly imparts flexibility to the chain. Pro imparts rigidity to the chain and disrupts α helices. These residues may be essential in certain regions of the polypeptide, but substitutable elsewhere.

One, two or three conservative substitutions are more likely to be tolerated than a larger number.

"Semi-conservative substitutions" are defined herein as being substitutions within supergroup I/II/III or within supergroup IV/V, but not within a single one of groups I-V. They also include replacement of any other amino acid with

alanine. If a substitution is not conservative, it preferably is semi-conservative.

"Non-conservative substitutions" are substitutions which are not "conservative" or "semi-conservative".

"Highly conservative substitutions" are a subset of conservative substitutions, and are exchanges of amino acids within the groups Phe/Tyr/Trp, Met/Leu/Ile/Val, His/Arg/Lys, Asp/Glu and Ser/Thr/Ala. They are more likely to be tolerated than other conservative substitutions. Again, the smaller the number of substitutions, the more likely they are to be tolerated.

"Conservatively Identical"

A protein (peptide) is conservatively identical to a reference protein (peptide) it differs from the latter, if at all, solely by conservative modifications, the protein (peptide remaining at least seven amino acids long if the reference protein (peptide) was at least seven amino acids long.

A protein is at least semi-conservatively identical to a reference protein (peptide) if it differs from the latter, if at all, solely by semi-conservative or conservative modifications.

A protein (peptide) is nearly conservatively identical to a reference protein (peptide) if it differs from the latter, if at all, solely by one or more conservative modifications and/or a single nonconservative substitution.

It is highly conservatively identical if it differs, if at all, solely by highly conservative substitutions. Highly conservatively identical proteins are preferred to those merely conservatively identical. An absolutely identical protein is even more preferred.

The core sequence of a reference protein (peptide) is the largest single fragment which retains at least 10% of a particular specific binding activity, if one is specified, or otherwise of at least one specific binding activity of

the referent. If the referent has more than one specific binding activity, it may have more than one core sequence, and these may overlap or not.

If it is taught that a peptide of the present invention may have a particular similarity relationship (e.g., markedly identical) to a reference protein (peptide), preferred peptides are those which comprise a sequence having that relationship to a core sequence of the reference protein (peptide), but with internal insertions or deletions in either sequence excluded. Even more preferred peptides are those whose entire sequence has that relationship, with the same exclusion, to a core sequence of that reference protein (peptide).

Library

The term "library" generally refers to a collection of chemical or biological entities which are related in origin, structure, and/or function, and which can be screened simultaneously for a property of interest.

Libraries may be classified by how they are constructed (natural vs. artificial diversity; combinatorial vs. noncombinatorial), how they are screened (hybridization, expression, display), or by the nature of the screened library members (peptides, nucleic acids, etc.).

In a "natural diversity" library, essentially all of the diversity arose without human intervention. This would be true, for example, of messenger RNA extracted from a non-engineered cell.

In a "synthetic diversity" library, essentially all of the diversity arose deliberately as a result of human intervention. This would be true for example of a combinatorial library; note that a small level of natural diversity could still arise as a result of spontaneous mutation. It would also be true of a noncombinatorial library of compounds collected from diverse sources, even if they were all natural products.

In a "non-natural diversity" library, at least some of

the diversity arose deliberately through human intervention.

In a "controlled origin" library, the source of the diversity is limited in some way. A limitation might be to cells of a particular individual, to a particular species, or to a particular genus, or, more complexly, to individuals of a particular species who are of a particular age, sex, physical condition, geographical location, occupation and/or familial relationship. Alternatively or additionally, it might be to cells of a particular tissue or organ. Or it could be cells exposed to particular pharmacological, environmental, or pathogenic conditions. Or the library could be of chemicals, or a particular class of chemicals, produced by such cells.

In a "controlled structure" library, the library members are deliberately limited by the production conditions to particular chemical structures. For example, if they are oligomers, they may be limited in length and monomer composition, e.g. hexapeptides composed of the twenty genetically encoded amino acids.

Hybridization Library

In a hybridization library, the library members are nucleic acids, and are screened using a nucleic acid hybridization probe. Bound nucleic acids may then be amplified, cloned, and/or sequenced.

Expression Library

In an expression library, the screened library members are gene expression products, but one may also speak of an underlying library of genes encoding those products. The library is made by subcloning DNA encoding the library members (or portions thereof) into expression vectors (or into cloning vectors which subsequently are used to construct expression vectors), each vector comprising an expressible gene encoding a particular library member, introducing the expression vectors into suitable cells, and expressing the genes so the expression products are produced.

In one embodiment, the expression products are secreted, so the library can be screened using an affinity reagent, such as an antibody or receptor. The bound expression products may be sequenced directly, or their sequences inferred by, e.g., sequencing at least the variable portion of the encoding DNA.

In a second embodiment, the cells are lysed, thereby exposing the expression products, and the latter are screened with the affinity reagent.

In a third embodiment, the cells express the library members in such a manner that they are displayed on the surface of the cells, or on the surface of viral particles produced by the cells. (See display libraries, below).

In a fourth embodiment, the screening is not for the ability of the expression product to bind to an affinity reagent, but rather for its ability to alter the phenotype of the host cell in a particular detectable manner. Here, the screened library members are transformed cells, but there is a first underlying library of expression products which mediate the behavior of the cells, and a second underlying library of genes which encode those products.

Display Library

In a display library, the library members are each conjugated to, and displayed upon, a support of some kind. The support may be living (a cell or virus), or nonliving (e.g., a bead or plate).

If the support is a cell or virus, display will normally be effectuated by expressing a fusion protein which comprises the library member, a carrier moiety allowing integration of the fusion protein into the surface of the cell or virus, and optionally a lining moiety. In a variation on this theme, the cell coexpresses a first fusion comprising the library member and a linking moiety L1, and a second fusion comprising a linking moiety L2 and the carrier moiety. L1 and L2 interact to associate the first fusion with the second fusion and hence, indirectly, the library member with the surface of the cell or virus.

Soluble Library

In a soluble library, the library members are free in solution. A soluble library may be produced directly, or one may first make a display library and then release the library members from their supports.

Encapsulated Library

In an encapsulated library, the library members are inside cells or liposomes. Generally speaking, encapsulated libraries are used to store the library members for future use; the members are extracted in some way for screening purposes. However, if they differentially affect the phenotype of the cells, they may be screened indirectly by screening the cells.

cDNA Library

A cDNA library is usually prepared by extracting RNA from cells of particular origin, fractionating the RNA to isolate the messenger RNA (mRNA has a poly(A) tail, so this is usually done by oligo-dT affinity chromatography), synthesizing complementary DNA (cDNA) using reverse transcriptase, DNA polymerase, and other enzymes, subcloning the cDNA into vectors, and introducing the vectors into cells. Often, only mRNAs or cDNAs of particular sizes will be used, to make it more likely that the cDNA encodes a functional polypeptide.

A cDNA library explores the natural diversity of the transcribed DNAs of cells from a particular source. It is not a combinatorial library.

A cDNA library may be used to make a hybridization library, or it may be used as an (or to make) expression library.

Genomic DNA Library

A genomic DNA library is made by extracting DNA from a particular source, fragmenting the DNA, isolating fragments of a particular size range, subcloning the DNA fragments into vectors, and introducing the vectors into cells.

Like a cDNA library, a genomic DNA library is a natural diversity library, and not a combinatorial library. A genomic DNA library may be used the same way as a cDNA library.

Synthetic DNA library

A synthetic DNA library may be screened directly (as a hybridization library), or used in the creation of an expression or display library of peptides/proteins.

Combinatorial Libraries

The term "combinatorial library" refers to a library in which the individual members are either systematic or random combinations of a limited set of basic elements, the properties of each member being dependent on the choice and location of the elements incorporated into it. Typically, the members of the library are at least capable of being screened simultaneously. Randomization may be complete or partial; some positions may be randomized and others predetermined, and at random positions, the choices may be limited in a predetermined manner. The members of a combinatorial library may be oligomers or polymers of some kind, in which the variation occurs through the choice of monomeric building block at one or more positions of the oligomer or polymer, and possibly in terms of the connecting linkage, or the length of the oligomer or polymer, too. Or the members may be nonoligomeric molecules with a standard core structure, like the 1,4-benzodiazepine structure, with the variation being introduced by the choice of substituents at particular variable sites on the core structure. Or the members may be nonoligomeric molecules assembled like a jigsaw puzzle, but wherein each piece has both one or more variable moieties (contributing to library diversity) and one or more constant moieties (providing the functionalities for coupling the piece in question to other pieces).

Thus, in a typical combinatorial library, chemical building blocks are at least partially randomly combined into a large number (as high as 10^{15}) of different compounds,

which are then simultaneously screened for binding (or other) activity against one or more targets.

In a "simple combinatorial library", all of the members belong to the same class of compounds (e.g., peptides) and can be synthesized simultaneously. A "composite combinatorial library" is a mixture of two or more simple libraries, e.g., DNAs and peptides, or peptides, peptoids, and PNAs, or benzodiazepines and carbamates. The number of component simple libraries in a composite library will, of course, normally be smaller than the average number of members in each simple library, as otherwise the advantage of a library over individual synthesis is small.

Libraries of thousands, even millions, of random oligopeptides have been prepared by chemical synthesis (Houghten *et al.*, Nature, 354:84-6(1991)), or gene expression (Marks *et al.*, J Mol Biol, 222:581-97(1991)), displayed on chromatographic supports (Lam *et al.*, Nature, 354:82-4(1991)), inside bacterial cells (Colas *et al.*, Nature, 380:548-550(1996)), on bacterial pili (Lu, Bio/Technology, 13:366-372(1990)), or phage (Smith, Science, 228:1315-7(1985)), and screened for binding to a variety of targets including antibodies (Valadon *et al.*, J Mol Biol, 261:11-22(1996)), cellular proteins (Schmitz *et al.*, J Mol Biol, 260:664-677(1996)), viral proteins (Hong and Boulanger, Embo J, 14:4714-4727(1995)), bacterial proteins (Jacobsson and Frykberg, Biotechniques, 18:878-885(1995)), nucleic acids (Cheng *et al.*, Gene, 171:1-8(1996)), and plastic (Siani *et al.*, J Chem Inf Comput Sci, 34:588-593(1994)).

Libraries of proteins (Ladner, USP 4,664,989), peptoids (Simon *et al.*, Proc Natl Acad Sci U S A, 89:9367-71(1992)), nucleic acids (Ellington and Szostak, Nature, 246:818(1990)), carbohydrates, and small organic molecules (Eichler *et al.*, Med Res Rev, 15:481-96(1995)) have also been prepared or suggested for drug screening purposes.

The first combinatorial libraries were composed of peptides or proteins, in which all or selected amino acid positions were randomized. Peptides and proteins can exhibit

high and specific binding activity, and can act as catalysts. In consequence, they are of great importance in biological systems.

Nucleic acids have also been used in combinatorial libraries. Their great advantage is the ease with which a nucleic acid with appropriate binding activity can be amplified. As a result, combinatorial libraries composed of nucleic acids can be of low redundancy and hence, of high diversity.

There has also been much interest in combinatorial libraries based on small molecules, which are more suited to pharmaceutical use, especially those which, like benzodiazepines, belong to a chemical class which has already yielded useful pharmacological agents. The techniques of combinatorial chemistry have been recognized as the most efficient means for finding small molecules that act on these targets. At present, small molecule combinatorial chemistry involves the synthesis of either pooled or discrete molecules that present varying arrays of functionality on a common scaffold. These compounds are grouped in libraries that are then screened against the target of interest either for binding or for inhibition of biological activity.

The size of a library is the number of molecules in it. The simple diversity of a library is the number of unique structures in it. There is no formal minimum or maximum diversity. If the library has a very low diversity, the library has little advantage over just synthesizing and screening the members individually. If the library is of very high diversity, it may be inconvenient to handle, at least without automatizing the process. The simple diversity of a library is preferably at least 10, 10E2, 10E3, 10E4, 10E6, 10E7, 10E8 or 10E9, the higher the better under most circumstances. The simple diversity is usually not more than 10E15, and more usually not more than 10E10. The average sampling level is the size divided by the simple diversity. The expected average sampling level must be high enough to provide a reasonable assurance that, if a given

structure were expected, as a consequence of the library design, to be present, that the actual average sampling level will be high enough so that the structure, if satisfying the screening criteria, will yield a positive result when the library is screened. Thus, the preferred average sampling level is a function of the detection limit, which in turn is a function of the strength of the signal to be screened.

There are more complex measures of diversity than simple diversity. These attempt to take into account the degree of structural difference between the various unique sequences. These more complex measures are usually used in the context of small organic compound libraries, see below.

The library members may be presented as solutes in solution, or immobilized on some form of support. In the latter case, the support may be living (cell, virus) or nonliving (bead, plate, etc.). The supports may be separable (cells, virus particles, beads) so that binding and nonbinding members can be separated, or nonseparable (plate). In the latter case, the members will normally be placed on addressable positions on the support. The advantage of a soluble library is that there is no carrier moiety that could interfere with the binding of the members to the support. The advantage of an immobilized library is that it is easier to identify the structure of the members which were positive.

When screening a soluble library, or one with a separable support, the target is usually immobilized. When screening a library on a nonseparable support, the target will usually be labeled.

Oligonucleotide Libraries

An oligonucleotide library is a combinatorial library, at least some of whose members are single-stranded oligonucleotides having three or more nucleotides connected by phosphodiester or analogous bonds. The oligonucleotides may be linear, cyclic or branched, and may include non-nucleic acid moieties. The nucleotides are not limited to the nucleotides normally found in DNA or RNA. For examples

of nucleotides modified to increase nuclease resistance and chemical stability of aptamers, see Chart 1 in Osborne and Ellington, Chem. Rev., 97: 349-70 (1997). For screening of RNA, see Ellington and Szostak, Nature, 346: 818-22 (1990).

There is no formal minimum or maximum size for these oligonucleotides. However, the number of conformations which an oligonucleotide can assume increases exponentially with its length in bases. Hence, a longer oligonucleotide is more likely to be able to fold to adapt itself to a protein surface. On the other hand, while very long molecules can be synthesized and screened, unless they provide a much superior affinity to that of shorter molecules, they are not likely to be found in the selected population, for the reasons explained by Osborne and Ellington (1997). Hence, the libraries of the present invention are preferably composed of oligonucleotides having a length of 3 to 100 bases, more preferably 15 to 35 bases. The oligonucleotides in a given library may be of the same or of different lengths.

Oligonucleotide libraries have the advantage that libraries of very high diversity (e.g., 10^{15}) are feasible, and binding molecules are readily amplified in vitro by polymerase chain reaction (PCR). Moreover, nucleic acid molecules can have very high specificity and affinity to targets.

In a preferred embodiment, this invention prepares and screens oligonucleotide libraries by the SELEX method, as described in King and Famulok, Molec. Biol. Repts., 20: 97-107 (1994); L. Gold, C. Tuerk. Methods of producing nucleic acid ligands, US#5595877; Oliphant et al. Gene 44:177 (1986).

The term "aptamer" is conferred on those oligonucleotides which bind the target protein. Such aptamers may be used to characterize the target protein, both directly (through identification of the aptamer and the points of contact between the aptamer and the protein) and indirectly (by use of the aptamer as a ligand to modify the chemical reactivity of the protein).

In a classic oligonuclotide, each nucleotide (monomeric unit) is composed of a phosphate group, a sugar moiety, and either a purine or a pyrimidine base. In DNA, the sugar is deoxyribose and in RNA it is ribose. The nucleotides are linked by 5'-3' phosphodiester bonds.

The deoxyribose phosphate backbone of DNA can be modified to increase resistance to nuclease and to increase penetration of cell membranes. Derivatives such as mono- or dithiophosphates, methyl phosphonates, boranophosphates, formacetals, carbamates, siloxanes, and dimethylenethio- -sulfoxideo- and-sulfono- linked species are known in the art.

Peptide Library

A peptide is composed of a plurality of amino acid residues joined together by peptidyl (-NHCO-) bonds. A biogenic peptide is a peptide in which the residues are all genetically encoded amino acid residues; it is not necessary that the biogenic peptide actually be produced by gene expression.

Amino acids are the basic building blocks with which peptides and proteins are constructed. Amino acids possess both an amino group ($-NH_2$) and a carboxylic acid group (-COOH). Many amino acids, but not all, have the alpha amino acid structure NH_2-CHR-COOH, where R is hydrogen, or any of a variety of functional groups.

Twenty amino acids are genetically encoded: Alanine, Arginine, Asparagine, Aspartic Acid, Cysteine, Glutamic Acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, and Valine. Of these, all save Glycine are optically isomeric, however, only the L-form is found in humans. Nevertheless, the D-forms of these amino acids do have biological significance; D-Phe, for example, is a known analgesic.

Many other amino acids are also known, including: 2-Aminoadipic acid; 3-Aminoadipic acid; beta-Aminopropionic acid; 2-Aminobutyric acid; 4-Aminobutyric acid (Piperidinic

acid);6-Aminocaproic acid; 2-Aminoheptanoic acid; 2-Aminoisobutyric acid, 3-Aminoisobutyric acid; 2-Aminopimelic acid; 2,4-Diaminobutyric acid; Desmosine; 2,2'-Diaminopimelic acid; 2,3-Diaminopropionic acid; N-Ethylglycine; N-Ethylasparagine; Hydroxylysine; allo-Hydroxylysine; 3-Hydroxyproline; 4-Hydroxyproline; Isodesmosine; allo-Isoleucine; N-Methylglycine (Sarcosine); N-Methylisoleucine; N-Methylvaline; Norvaline; Norleucine; and Ornithine.

Peptides are constructed by condensation of amino acids and/or smaller peptides. The amino group of one amino acid (or peptide) reacts with the carboxylic acid group of a second amino acid (or peptide) to form a peptide (-NHCO-) bond, releasing one molecule of water. Therefore, when an amino acid is incorporated into a peptide, it should, technically speaking, be referred to as an amino acid residue. The core of that residue is the moiety which excludes the -NH and -CO linking functionalities which connect it to other residues. This moiety consists of one or more main chain atoms (see below) and the attached side chains.

The main chain moiety of each amino acid consists of the -NH and -CO linking functionalities and a core main chain moiety. Usually the latter is a single carbon atom. However, the core main chain moiety may include additional carbon atoms, and may also include nitrogen, oxygen or sulfur atoms, which together form a single chain. In a preferred embodiment, the core main chain atoms consist solely of carbon atoms.

The side chains are attached to the core main chain atoms. For alpha amino acids, in which the side chain is attached to the alpha carbon, the C-1, C-2 and N-2 of each residue form the repeating unit of the main chain, and the word "side chain" refers to the C-3 and higher numbered carbon atoms and their substituents. It also includes H atoms attached to the main chain atoms.

Amino acids may be classified according to the number of carbon atoms which appear in the main chain between the

carbonyl carbon and amino nitrogen atoms which participate in the peptide bonds. Among the 150 or so amino acids which occur in nature, alpha, beta, gamma and delta amino acids are known. These have 1-4 intermediary carbons. Only alpha amino acids occur in proteins. Proline is a special case of an alpha amino acid; its side chain also binds to the peptide bond nitrogen.

For beta and higher order amino acids, there is a choice as to which main chain core carbon a side chain other than H is attached to. The preferred attachment site is the C-2 (alpha) carbon, i.e., the one adjacent to the carboxyl carbon of the -CO linking functionality. It is also possible for more than one main chain atom to carry a side chain other than H. However, in a preferred embodiment, only one main chain core atom carries a side chain other than H.

A main chain carbon atom may carry either one or two side chains; one is more common. A side chain may be attached to a main chain carbon atom by a single or a double bond; the former is more common.

A simple combinatorial peptide library is one whose members are peptides having three or more amino acids connected via peptide bonds.

The peptides may be linear, branched, or cyclic, and may covalently or noncovalently include nonpeptidyl moieties. The amino acids are not limited to the naturally occurring or to the genetically encoded amino acids.

A biased peptide library is one in which one or more (but not all) residues of the peptides are constant residues.

Cyclic Peptides

Many naturally occurring peptides are cyclic. Cyclization is a common mechanism for stabilization of peptide conformation thereby achieving improved association of the peptide with its ligand and hence improved biological activity. Cyclization is usually achieved by intra-chain cystine formation, by formation of peptide bond between side chains or between N- and C- terminals. Cyclization was

usually achieved by peptides in solution, but several publications have appeared that describe cyclization of peptides on beads.

A peptide library may be an oligopeptide library or a protein library.

Oligopeptides

Preferably, the oligopeptides are at least five, six, seven or eight amino acids in length. Preferably, they are composed of less than 50, more preferably less than 20 amino acids.

In the case of an oligopeptide library, all or just some of the residues may be variable. The oligopeptide may be unconstrained, or constrained to a particular conformation by, e.g., the participation of constant cysteine residues in the formation of a constraining disulfide bond.

Proteins

Proteins, like oligopeptides, are composed of a plurality of amino acids, but the term protein is usually reserved for longer peptides, which are able to fold into a stable conformation. A protein may be composed of two or more polypeptide chains, held together by covalent or noncovalent crosslinks. These may occur in a homooligomeric or a heterooligomeric state.

A peptide is considered a protein if it (1) is at least 50 amino acids long, or (2) has at least two stabilizing covalent crosslinks (e.g., disulfide bonds). Thus, conotoxins are considered proteins.

Usually, the proteins of a protein library will be characterizable as having both constant residues (the same for all proteins in the library) and variable residues (which vary from member to member). This is simply because, for a given range of variation at each position, the sequence space (simple diversity) grows exponentially with the number of residue positions, so at some point it becomes inconvenient for all residues of a peptide to be variable

positions. Since proteins are usually larger than oligopeptides, it is more common for protein libraries than oligopeptide libraries to feature variable positions.

In the case of a protein library, it is desirable to focus the mutations at those sites which are tolerant of mutation. These may be determined by alanine scanning mutagenesis or by comparison of the protein sequence to that of homologous proteins of similar activity. It is also more likely that mutation of surface residues will directly affect binding. Surface residues may be determined by inspecting a 3D structure of the protein, or by labeling the surface and then ascertaining which residues have received labels. They may also be inferred by identifying regions of high hydrophilicity within the protein.

Because proteins are often altered at some sites but not others, protein libraries can be considered a special case of the biased peptide library.

There are several reasons that one might screen a protein library instead of an oligopeptide library, including (1) a particular protein, mutated in the library, has the desired activity to some degree already, and (2) the oligopeptides are not expected to have a sufficiently high affinity or specificity since they do not have a stable conformation.

When the protein library is based on a parental protein which does not have the desired activity, the parental protein will usually be one which is of high stability (melting point >= 50 deg. C.) and/or possessed of hypervariable regions.

The variable domains of an antibody possess hypervariable regions and hence, in some embodiments, the protein library comprises members which comprise a mutant of VH or VL chain, or a mutant of an antigen-specific binding fragment of such a chain. VH and VL chains are usually each about 110 amino acid residues, and are held in proximity by a disulfide bond between the adjoing CL and CH1 regions to form a variable domain. Together, the VH, VL, CL and CH1 form an Fab fragment.

In human heavy chains, the hypervariable regions are at 31-35, 49-65, 98-111 and 84-88, but only the first three are involved in antigen binding. There is variation among VH and VL chains at residues outside the hypervariable regions, but to a much lesser degree.

A sequence is considered a mutant of a VH or VL chain if it is at least 80% identical to a naturally occurring VH or VL chain at all residues outside the hypervariable region.

In a preferred embodiment, such antibody library members comprise both at least one VH chain and at least one VL chain, at least one of which is a mutant chain, and which chains may be derived from the same or different antibodies. The VH and VL chains may be covalently joined by a suitable linker moiety, as in a "single chain antibody", or they may be noncovalently joined, as in a naturally occurring variable domain.

If the joining is noncovalent, and the library is displayed on cells or virus, then either the VH or the VL chain may be fused to the carrier surface/coat protein. The complementary chain may be co-expressed, or added exogenously to the library.

The members may further comprise some or all of an antibody constant heavy and/or constant light chain, or a mutant thereof.

Peptoid Library

A peptoid is an analogue of a peptide in which one or more of the peptide bonds (-NH-CO-) are replaced by pseudopeptide bonds, which may be the same or different. It is not necessary that all of the peptide bonds be replaced, i.e., a peptoid may include one or more conventional amino acid residues, e.g., proline.

A peptide bond has two small divalent linker elements, -NH- and -CO-. Thus, a preferred class of psuedopeptide bonds are those which consist of two small divalent linker elements. Each may be chosen independently from the group consisting of amine (-NH-), substituted amine (-NR-),

carbonyl (-CO-), thiocarbonyl (-CS-),methylene (-CH2-), monosubstituted methylene (-CHR-), disubstituted methylene (-CR1R2-), ether (-O-) and thioether (-S-). The more preferred pseudopeptide bonds include:

N-modified -NRCO-
Carba Ψ $-CH_2-CH_2-$
Depsi Ψ -CO-O-
Hydroxyethylene Ψ $-CHOH-CH_2-$
Ketomethylene Ψ $-CO-CH_2-$
Methylene-Oxy $-CH_2-O-$
Reduced $-CH_2-NH-$
Thiomethylene $-CH_2-S-$
Thiopeptide -CS-NH-
Retro-Inverso -CO-NH-

A single peptoid molecule may include more than one kind of pseudopeptide bond.

For the purposes of introducing diversity into a peptoid library, one may vary (1) the side chains attached to the core main chain atoms of the monomers linked by the pseudopeptide bonds, and/or (2) the side chains (e.g., the -R of an -NRCO-) of the pseudopeptide bonds. Thus, in one embodiment, the monomeric units which are not amino acid residues are of the structure -NR1-CR2-CO-, where at least one of R1 and R2 are not hydrogen. If there is variability in the pseudopeptide bond, this is most conveniently done by using an -NRCO- or other pseudopeptide bond with an R group, and varying the R group. In this event, the R group will usually be any of the side chains characterizing the amino acids of peptides, as previously discussed.

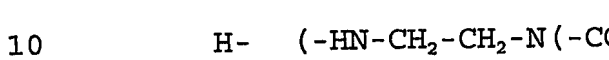
If the R group of the pseudopeptide bond is not variable, it will usually be small, e.g., not more than 10 atoms (e.g., hydroxyl, amino, carboxyl, methyl, ethyl, propyl).

If the conjugation chemistries are compatible, a simple combinatorial library may include both peptides and peptoids.

Peptide Nucleic Acid Library

A PNA oligomer is here defined as one comprising a plurality of units, at least one of which is a PNA monomer which comprises a side chain comprising a nucleobase. For nucleobases, see USP 6,077,835.

The classic PNA oligomer is composed of (2-aminoethyl)glycine units, with nucleobases attached by methylene carbonyl linkers. That is, it has the structure



where the outer parenthesized substructure is the PNA monomer.

In this structure, the nucleobase B is separated from the backbone N by three bonds, and the points of attachment of the side chains are separated by six bonds. The nucleobase may be any of the bases included in the nucleotides discussed in connection with oligonucleotide libraries. The bases of nucleotides A, G, T, C and U are preferred.

A PNA oligomer may further comprise one or more amino acid residues, especially glycine and proline.

One can readily envision related molecules in which (1) the -COCH2- linker is replaced by another linker, especially one composed of two small divalent linkers as defined previously, (2) a side chain is attached to one of the three main chain carbons not participating in the peptide bond (either instead or in addition to the side chain attached to the N of the classic PNA); and/or (3) the peptide bonds are replaced by pseudopeptide bonds as disclosed previously in the context of peptoids.

PNA oligomer libraries have been made; see e.g. Cook, 6,204,326.

Small Organic Compound Library

The small organic compound library ("compound library", for short) is a combinatorial library whose members are

suitable for use as drugs if, indeed, they have the ability to mediate a biological activity of the target protein.

Peptides have certain disadvantages as drugs. These include susceptibility to degradation by serum proteases, and difficulty in penetrating cell membranes. Preferably, all or most of the compounds of the compound library avoid, or at least do not suffer to the same degree, one or more of the pharmaceutical disadvantages of peptides.

In designing a compound library, it is helpful to bear in mind the methods of molecular modification typically used to obtain new drugs. Three basic kinds of modification may be identified: disjunction, in which a lead drug is simplified to identify its component pharmacophoric moieties; conjunction, in which two or more known pharmacophoric moieties, which may be the same or different, are associated, covalently or noncovalently, to form a new drug; and alteration, in which one moiety is replaced by another which may be similar or different, but which is not in effect a disjunction or conjunction. The use of the terms "disjunction", "conjunction" and "alteration" is intended only to connote the structural relationship of the end product to the original leads, and not how the new drugs are actually synthesized, although it is possible that the two are the same.

The process of disjunction is illustrated by the evolution of neostigmine (1931) and edrophonium (1952) from physostigmine (1925). Subsequent conjunction is illustrated by demecarium (1956) and ambenonium (1956).

Alterations may modify the size, polarity, or electron distribution of an original moiety. Alterations include ring closing or opening, formation of lower or higher homologues, introduction or saturation of double bonds, introduction of optically active centers, introduction, removal or replacement of bulky groups, isosteric or bioisosteric substitution, changes in the position or orientation of a group, introduction of alkylating groups, and introduction, removal or replacement of groups with a view toward inhibiting or promoting inductive

(electrostatic) or conjugative (resonance) effects.

Thus, the substituents may include electron acceptors and/or electron donors. Typical electron donors (+I) include $-CH_3$, $-CH_2R$, $-CHR_2$, $-CR_3$ and $-COO^-$. Typical electron acceptors (-I) include $-NH_3+$, $-NR_3+$, $-NO_2$, -CN, -COOH, -COOR, -CHO, -COR, -COR, -F, -C1, -Br, -OH, -OR, -SH, -SR, $-CH=CH_2$, $-CR=CR_2$, and -C=CH.

The substituents may also include those which increase or decrease electronic density in conjugated systems. The former (+R) groups include $-CH_3$, $-CR_3$, -F, -C1, -Br, -I, -OH, -OR, -OCOR, -SH, -SR, $-NH_2$, $-NR_2$, and -NHCOR. The later (-R) groups include $-NO_2$, -CN, -CHC, -COR, -COOH, -COOR, $-CONH_2$, $-SO_2R$ and $-CF_3$.

Synthetically speaking, the modifications may be achieved by a variety of unit processes, including nucleophilic and electrophilic substitution, reduction and oxidation, addition elimination, double bond cleavage, and cyclization.

For the purpose of constructing a library, a compound, or a family of compounds, having one or more pharmacological activities (which need not be related to the known or suspected activities of the target protein), may be disjoined into two or more known or potential pharmacophoric moieties. Analogues of each of these moieties may be identified, and mixtures of these analogues reacted so as to reassemble compounds which have some similarity to the original lead compound. It is not necessary that all members of the library possess moieties analogous to all of the moieties of the lead compound.

The design of a library may be illustrated by the example of the benzodiazepines. Several benzodiazepine drugs, including chlordiazepoxide, diazepam and oxazepam, have been used as anti-anxiety drugs. Derivatives of benzodiazepines have widespread biological activities; derivatives have been reported to act not only as anxiolytics, but also as anticonvulsants; cholecystokinin (CCK) receptor subtype A or B, kappa opioid receptor, platelet activating factor, and HIV transactivator Tat

antagonists, and GPIIbIIa, reverse transcriptase and ras farnesyltransferase inhibitors.

The benzodiazepine structure has been disjoined into a 2-aminobenzophenone, an amino acid, and an alkylating agent. See Bunin, et al., Proc. Nat. Acad. Sci. USA, 91:4708 (1994). Since only a few 2-aminobenzophenone derivatives are commercially available, it was later disjoined into 2-aminoarylstannane, an acid chloride, an amino acid, and an alkylating agent. Bunin, et al., Meth. Enzymol., 267:448 (1996). The arylstannane may be considered the core structure upon which the other moieties are substituted, or all four may be considered equals which are conjoined to make each library member.

A basic library synthesis plan and member structure is shown in Figure 1 of Fowlkes, et al., U.S. Serial No. 08/740,671, incorporated by reference in its entirety. The acid chloride building block introduces variability at the R^1 site. The R^2 site is introduced by the amino acid, and the R^3 site by the alkylating agent. The R^4 site is inherent in the arylstannane. Bunin, et al. generated a 1, 4-benzodiazepine library of 11,200 different derivatives prepared from 20 acid chlorides, 35 amino acids, and 16 alkylating agents. (No diversity was introduced at R^4; this group was used to couple the molecule to a solid phase.) According to the Available Chemicals Directory (HDL Information Systems, San Leandro CA), over 300 acid chlorides, 80 Fmoc-protected amino acids and 800 alkylating agents were available for purchase (and more, of course, could be synthesized). The particular moieties used were chosen to maximize structural dispersion, while limiting the numbers to those conveniently synthesized in the wells of a microtiter plate. In choosing between structurally similar compounds, preference was given to the least substituted compound.

The variable elements included both aliphatic and aromatic groups. Among the aliphatic groups, both acyclic and cyclic (mono- or poly-) structures, substituted or not, were tested. (While all of the acyclic groups were linear,

it would have been feasible to introduce a branched aliphatic). The aromatic groups featured either single and multiple rings, fused or not, substituted or not, and with heteroatoms or not. The secondary substitutents included -NH_2, -OH, -OMe, -CN, -Cl, -F, and -COOH. While not used, spacer moieties, such as -O-, -S-, -OO-, -CS-, -NH-, and -NR-, could have been incorporated.

Bunin et al. suggest that instead of using a 1, 4-benzodiazepine as a core structure, one may instead use a 1, 4-benzodiazepine-2, 5-dione structure.

As noted by Bunin et al., it is advantageous, although not necessary, to use a linkage strategy which leaves no trace of the linking functionality, as this permits construction of a more diverse library.

Other combinatorial nonoligomeric compound libraries known or suggested in the art have been based on carbamates, mercaptoacylated pyrrolidines, phenolic agents, aminimides, N-acylamino ethers (made from amino alcohols, aromatic hydroxy acids, and carboxylic acids), N-alkylamino ethers (made from aromatic hydroxy acids, amino alcohols and aldehydes) 1, 4-piperazines, and 1, 4-piperazine-6-ones.

DeWitt, et al., Proc. Nat. Acad. Sci. (USA), 90:6909-13 (1993) describe the simultaneous but separate, synthesis of 40 discrete hydantoins and 40 discrete benzodiazepines. They carry out their synthesis on a solid support (inside a gas dispersion tube), in an array format, as opposed to other conventional simultaneous synthesis techniques (e.g., in a well, or on a pin). The hydantoins were synthesized by first simultaneously deprotecting and then treating each of five amino acid resins with each of eight isocyanates. The benzodiazepines were synthesized by treating each of five deprotected amino acid resins with each of eight 2-amino benzophenone imines.

Chen, et al., J. Am. Chem. Soc., 116:2661-62 (1994) described the preparation of a pilot (9 member) combinatorial library of formate esters. A polymer bead-bound aldehyde preparation was "split" into three aliquots, each reacted with one of three different ylide reagents.

The reaction products were combined, and then divided into three new aliquots, each of which was reacted with a different Michael donor. Compound identity was found to be determinable on a single bead basis by gas chromatography/mass spectroscopy analysis.

Holmes, USP 5,549,974 (1996) sets forth methodologies for the combinatorial synthesis of libraries of thiazolidinones and metathiazanones. These libraries are made by combination of amines, carbonyl compounds, and thiols under cyclization conditions.

Ellman, USP 5,545,568 (1996) describes combinatorial synthesis of benzodiazepines, prostaglandins, beta-turn mimetics, and glycerol-based compounds. See also Ellman, USP 5,288,514.

Summerton, USP 5,506,337 (1996) discloses methods of preparing a combinatorial library formed predominantly of morpholino subunit structures.

Heterocylic combinatorial libraries are reviewed generally in Nefzi, et al., Chem. Rev., 97:449-472 (1997).

For pharmacological classes, see, e.g., Goth, Medical Pharmacology: Principles and Concepts (C.V. Mosby Co.: 8th ed. 1976); Korolkovas and Burckhalter, Essentials of Medicinal Chemistry (John Wiley & Sons, Inc.: 1976). For synthetic methods, see, e.g., Warren, Organic Synthesis: The Disconnection Approach (John Wiley & Sons, Ltd.: 1982); Fuson, Reactions of Organic Compounds (John Wiley & Sons: 1966); Payne and Payne, How to do an Organic Synthesis (Allyn and Bacon, Inc.: 1969); Greene, Protective Groups in Organic Synthesis (Wiley-Interscience). For selection of substituents, see e.g., Hansch and Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology (John Wiley & Sons: 1979).

The library is preferably synthesized so that the individual members remain identifiable so that, if a member is shown to be active, it is not necessary to analyze it. Several methods of identification have been proposed, including:

(1) encoding, i.e., the attachment to each member of an identifier moiety which is more readily identified than the member proper. This has the disadvantage that the tag may itself influence the activity of the conjugate.

(2) spatial addressing, e.g., each member is synthesized only at a particular coordinate on or in a matrix, or in a particular chamber. This might be, for example, the location of a particular pin, or a particular well on a microtiter plate, or inside a "tea bag".

The present invention is not limited to any particular form of identification.

However, it is possible to simply characterize those members of the library which are found to be active, based on the characteristic spectroscopic indicia of the various building blocks.

Solid phase synthesis permits greater control over which derivatives are formed. However, the solid phase could interfere with activity. To overcome this problem, some or all of the molecules of each member could be liberated, after synthesis but before screening.

Examples of candidate simple libraries which might be evaluated include derivatives of the following:

Cyclic Compounds Containing One Hetero Atom

Heteronitrogen

pyrroles

pentasubstituted pyrroles

pyrrolidines

pyrrolines

prolines

indoles

beta-carbolines

pyridines

dihydropyridines

1,4-dihydropyridines

pyrido[2,3-d]pyrimidines

tetrahydro-3H-imidazo[4,5-c] pyridines

Isoquinolines
tetrahydroisoquinolines
quinolones
beta-lactams
azabicyclo[4.3.0]nonen-8-one amino acid
Heterooxygen
furans
tetrahydrofurans
2,5-disubstituted tetrahydrofurans
pyrans
hydroxypyranones
tetrahydroxypyranones
gamma-butyrolactones
Heterosulfur
sulfolenes
Cyclic Compounds with Two or More Hetero atoms
Multiple heteronitrogens
imidazoles
pyrazoles
piperazines
diketopiperazines
arylpiperazines
benzylpiperazines
benzodiazepines
1,4-benzodiazepine-2,5-diones
hydantoins
5-alkoxyhydantoins
dihydropyrimidines
1,3-disubstituted-5,6-dihydopyrimidine-2,4-diones
cyclic ureas
cyclic thioureas
quinazolines
chiral 3-substituted-quinazoline-2,4-diones
triazoles
1,2,3-triazoles

purines
Heteronitrogen and Heterooxygen
dikelomorpholines
isoxazoles
isoxazolines
Heteronitrogen and Heterosulfur
thiazolidines
N-axylthiazolidines
dihydrothiazoles
2-methylene-2,3-dihydrothiazates
2-aminothiazoles
thiophenes
3-amino thiophenes
4-thiazolidinones
4-melathiazanones
benzisothiazolones

For details on synthesis of libraries, see Nefzi, et al., Chem. Rev., 97:449-72 (1997), and references cited therein.

Pharmaceutical Methods and Preparations

The preferred animal subject of the present invention is a mammal. By the term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects, although it is intended for veterinary and nutritional uses as well. Preferred nonhuman subjects are of the orders Primata (e.g., apes and monkeys), Artiodactyla or Perissodactyla (e.g., cows, pigs, sheep, horses, goats), Carnivora (e.g., cats, dogs), Rodenta (e.g., rats, mice, guinea pigs, hamsters), Lagomorpha (e.g., rabbits) or other pet, farm or laboratory mammals.

The term "protection", as used herein, is intended to include "prevention," "suppression" and "treatment." "Prevention", strictly speaking, involves administration of the pharmaceutical prior to the induction of the disease (or other adverse clinical condition). "Suppression" involves administration of the composition prior to the clinical

appearance of the disease. "Treatment" involves administration of the protective composition after the appearance of the disease.

It will be understood that in human and veterinary medicine, it is not always possible to distinguish between "preventing" and "suppressing" since the ultimate inductive event or events may be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or events. Therefore, unless qualified, the term "prevention" will be understood to refer to both prevention in the strict sense, and to suppression.

The preventative or prophylactic use of a pharmaceutical involves identifying subjects who are at higher risk than the general population of contracting the disease, and administering the pharmaceutical to them in advance of the clinical appearance of the disease. The effectiveness of such use is measured by comparing the subsequent incidence or severity of the disease, or of particular symptoms of the disease, in the treated subjects against that in untreated subjects of the same high risk group.

While high risk factors vary from disease to disease, in general, these include (1) prior occurrence of the disease in one or more members of the same family, or, in the case of a contagious disease, in individuals with whom the subject has come into potentially contagious contact at a time when the earlier victim was likely to be contagious, (2) a prior occurrence of the disease in the subject, (3) prior occurrence of a related disease, or a condition known to increase the likelihood of the disease, in the subject; (4) appearance of a suspicious level of a marker of the disease, or a related disease or condition; (5) a subject who is immunologically compromised, e.g., by radiation treatment, HIV infection, drug use,, etc., or (6) membership in a particular group (e.g., a particular age, sex, race, ethnic group, etc.) which has been epidemiologically associated with that disease.

A prophylaxis or treatment may be curative, that is,

directed at the underlying cause of a disease, or ameliorative, that is, directed at the symptoms of the disease, especially those which reduce the quality of life.

It should also be understood that to be useful, the protection provided need not be absolute, provided that it is sufficient to carry clinical value. An agent which provides protection to a lesser degree than do competitive agents may still be of value if the other agents are ineffective for a particular individual, if it can be used in combination with other agents to enhance the level of protection, or if it is safer than competitive agents. It is desirable that there be a statistically significant ($p=0.05$ or less) improvement in the treated subject relative to an appropriate untreated control, and it is desirable that this improvement be at least 10%, more preferably at least 25%, still more preferably at least 50%, even more preferably at least 100%, in some indicia of the incidence or severity of the disease or of at least one symptom of the disease.

At least one of the drugs of the present invention may be administered, by any means that achieve their intended purpose, to protect a subject against a disease or other adverse condition. The form of administration may be systemic or topical. For example, administration of such a composition may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. Parenteral administration can be by bolus injection or by gradual perfusion over time.

A typical regimen comprises administration of an effective amount of the drug, administered over a period ranging from a single dose, to dosing over a period of hours, days, weeks, months, or years.

It is understood that the suitable dosage of a drug of the present invention will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can

be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This will typically involve adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

Prior to use in humans, a drug will first be evaluated for safety and efficacy in laboratory animals. In human clinical studies, one would begin with a dose expected to be safe in humans, based on the preclinical data for the drug in question, and on customary doses for analogous drugs (if any). If this dose is effective, the dosage may be decreased, to determine the minimum effective dose, if desired. If this dose is ineffective, it will be cautiously increased, with the patients monitored for signs of side effects. See, e.g., Berkow et al, eds., *The Merck Manual*, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman et al., eds., *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, *Pharmacology*, Little, Brown and Co., Boston, (1985), which references and references cited therein, are entirely incorporated herein by reference.

The total dose required for each treatment may be administered by multiple doses or in a single dose. The protein may be administered alone or in conjunction with other therapeutics directed to the disease or directed to other symptoms thereof.

The appropriate dosage form will depend on the disease, the pharmaceutical, and the mode of administration; possibilities include tablets, capsules, lozenges, dental pastes, suppositories, inhalants, solutions, ointments and parenteral depots. See, e.g., Berker, *supra*, Goodman, *supra*, Avery, *supra* and Ebadi, *supra*, which are entirely incorporated herein by reference, including all references cited therein.

In the case of peptide drugs, the drug may be

administered in the form of an expression vector comprising a nucleic acid encoding the peptide; such a vector, after incorporation into the genetic complement of a cell of the patient, directs synthesis of the peptide. Suitable vectors include genetically engineered poxviruses (vaccinia), adenoviruses, adeno-associated viruses, herpesviruses and lentiviruses which are or have been rendered nonpathogenic.

In addition to at least one drug as described herein, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. See, e.g., Berker, *supra*, Goodman, *supra*, Avery, *supra* and Ebadi, *supra*, which are entirely incorporated herein by reference, included all references cited therein.

Assay Compositions and Methods

Target Organism

The invention contemplates that it may be appropriate to ascertain or to mediate the biological activity of a substance of this invention in a target organism.

The target organism may be a plant, animal, or microorganism.

In the case of a plant, it may be an economic plant, in which case the drug may be intended to increase the disease, weather or pest resistance, alter the growth characteristics, or otherwise improve the useful characteristics or mute undesirable characteristics of the plant. Or it may be a weed, in which case the drug may be intended to kill or otherwise inhibit the growth of the plant, or to alter its characteristics to convert it from a weed to an economic plant. The plant may be a tree, shrub, crop, grass, etc. The plant may be an algae (which are in some cases also microorganisms), or a vascular plant, especially gymnosperms (particularly conifers) and angiosperms. Angiosperms may be monocots or dicots. The plants of greatest interest are rice, wheat, corn, alfalfa,

soybeans, potatoes, peanuts, tomatoes, melons, apples, pears, plums, pineapples, fir, spruce, pine, cedar, and oak.

If the target organism is a microorganism, it may be algae, bacteria, fungi, or a virus (although the biological activity of a virus must be determined in a virus-infected cell). The microorganism may be human or other animal or plant pathogen, or it may be nonpathogenic. It may be a soil or water organism, or one which normally lives inside other living things.

If the target organism is an animal, it may be a vertebrate or a nonvertebrate animal. Nonvertebrate animals are chiefly of interest when they act as pathogens or parasites, and the drugs are intended to act as biocidic or biostatic agents. Nonvertebrate animals of interest include worms, mollusks, and arthropods.

The target organism may also be a vertebrate animal, i.e., a mammal, bird, reptile, fish or amphibian. Among mammals, the target animal preferably belongs to the order Primata (humans, apes and monkeys), Artiodactyla (e.g., cows, pigs, sheep, goats, horses), Rodenta (e.g., mice, rats) Lagomorpha (e.g., rabbits, hares), or Carnivora (e.g., cats, dogs). Among birds, the target animals are preferably of the orders Anseriformes (e.g., ducks, geese, swans) or Galliformes (e.g., quails, grouse, pheasants, turkeys and chickens). Among fish, the target animal is preferably of the order Clupeiformes (e.g., sardines, shad, anchovies, whitefish, salmon).

Target Tissues

The term "target tissue" refers to any whole animal, physiological system, whole organ, part of organ, miscellaneous tissue, cell, or cell component (e.g., the cell membrane) of a target animal in which biological activity may be measured.

Routinely in mammals one would choose to compare and contrast the biological impact on virtually any and all tissues which express the subject receptor protein. The main tissues to use are: brain, heart, lung, kidney, liver,

pancreas, skin, intestines, adipose, stomach, skeletal muscle, adrenal glands, breast, prostate, vasculature, retina, cornea, thyroid gland, parathyroid glands, thymus, bone marrow, bone, etc.

Another classification would be by cell type: B cells, T cells, macrophages, neutrophils, eosinophils, mast cells, platelets, megakaryocytes, erythrocytes, bone marrow stomal cells, fibroblasts, neurons, astrocytes, neuroglia, microglia, epithelial cells (from any organ, e.g. skin, breast, prostate, lung, intestines etc), cardiac muscle cells, smooth muscle cells, striated muscle cells, osteoblasts, osteocytes, chondroblasts, chondrocytes, keratinocytes, melanocytes, etc.

Of course, in the case of a unicellular organism, there is no distinction between the "target organism" and the "target tissue".

Screening Assays

Assays intended to determine the binding or the biological activity of a substance are called preliminary screening assays.

Screening assays will typically be either in vitro (cell-free) assays (for binding to an immobilized receptor) or cell-based assays (for alterations in the phenotype of the cell). They will not involve screening of whole multicellular organisms, or isolated organs. The comments on diagnostic biological assays apply mutatis mutandis to screening cell-based assays.

In Vitro vs. In Vivo Assays

The term *in vivo* is descriptive of an event, such as binding or enzymatic action, which occurs within a living organism. The organism in question may, however, be genetically modified. The term *in vitro* refers to an event which occurs outside a living organism. Parts of an organism (e.g., a membrane, or an isolated biochemical) are used, together with artificial substrates and/or conditions. For the purpose of the present invention, the term in vitro

excludes events occurring inside or on an intact cell, whether of a unicellular or multicellular organism.

In vivo assays include both cell-based assays, and organismic assays. The cell-based assays include both assays on unicellular organisms, and assays on isolated cells or cell cultures derived from multicellular organisms. The cell cultures may be mixed, provided that they are not organized into tissues or organs. The term organismic assay refers to assays on whole multicellular organisms, and assays on isolated organs or tissues of such organisms.

In vitro Diagnostic Methods and Reagents

The in vitro assays of the present invention may be applied to any suitable analyte-containing sample, and may be qualitative or quantitative in nature.

Sample

The sample will normally be a biological fluid, such as blood, urine, lymph, semen, milk, or cerebrospinal fluid, or a fraction or derivative thereof, or a biological tissue, in the form of, e.g., a tissue section or homogenate. However, the sample conceivably could be (or derived from) a food or beverage, a pharmaceutical or diagnostic composition, soil, or surface or ground water. If a biological fluid or tissue, it may be taken from a human or other mammal, vertebrate or animal, or from a plant. The preferred sample is blood, or a fraction or derivative thereof.

Binding and Reaction Assays

The assay may be a binding assay, in which one step involves the binding of a diagnostic reagent to the analyte, or a reaction assay, which involves the reaction of a reagent with the analyte. The reagents used in a binding assay may be classified as to the nature of their interaction with analyte: (1) analyte analogues, or (2) analyte binding molecules (ABM). They may be labeled or insolubilized.

In a reaction assay, the assay may look for a direct reaction between the analyte and a reagent which is reactive with the analyte, or if the analyte is an enzyme or enzyme inhibitor, for a reaction catalyzed or inhibited by the analyte. The reagent may be a reactant, a catalyst, or an inhibitor for the reaction.

An assay may involve a cascade of steps in which the product of one step acts as the target for the next step. These steps may be binding steps, reaction steps, or a combination thereof.

Signal Producing System (SPS)

In order to detect the presence, or measure the amount, of an analyte, the assay must provide for a signal producing system (SPS) in which there is a detectable difference in the signal produced, depending on whether the analyte is present or absent (or, in a quantitative assay, on the amount of the analyte). The detectable signal may be one which is visually detectable, or one detectable only with instruments. Possible signals include production of colored or luminescent products, alteration of the characteristics (including amplitude or polarization) of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or product. The term "signal" is intended to include the discontinuance of an existing signal, or a change in the rate of change of an observable parameter, rather than a change in its absolute value. The signal may be monitored manually or automatically.

In a reaction assay, the signal is often a product of the reaction. In a binding assay, it is normally provided by a label borne by a labeled reagent.

Labels

The component of the signal producing system which is most intimately associated with the diagnostic reagent is called the "label". A label may be, e.g., a radioisotope, a

fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, an agglutinable particle.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention include ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, ^{32}P and ^{33}P. ^{125}I is preferred for antibody labeling.

The label may also be a fluorophore. When the fluorescently labeled reagent is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, fluorescence-emitting metals such as ^{125}Eu, or others of the lanthanide series, may be incorporated into a diagnostic reagent using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) of ethylenediamine-tetraacetic acid (EDTA).

The label may also be a chemiluminescent compound. The presence of the chemiluminescently labeled reagent is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used for labeling. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Enzyme labels, such as horseradish peroxidase and alkaline phosphatase, are preferred. When an enzyme label is used, the signal producing system must also include a

substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

An enzyme analyte may act as its own label if an enzyme inhibitor is used as a diagnostic reagent.

Binding Assay Formats

Binding assays may be divided into two basic types, heterogeneous and homogeneous. In heterogeneous assays, the interaction between the affinity molecule and the analyte does not affect the label, hence, to determine the amount or presence of analyte, bound label must be separated from free label. In homogeneous assays, the interaction does affect the activity of the label, and therefore analyte levels can be deduced without the need for a separation step.

In one embodiment, the ABM is insolubilized by coupling it to a macromolecular support, and analyte in the sample is allowed to compete with a known quantity of a labeled or specifically labelable analyte analogue. The "analyte analogue" is a molecule capable of competing with analyte for binding to the ABM, and the term is intended to include analyte itself. It may be labeled already, or it may be labeled subsequently by specifically binding the label to a moiety differentiating the analyte analogue from analyte. The solid and liquid phases are separated, and the labeled analyte analogue in one phase is quantified. The higher the level of analyte analogue in the solid phase, i.e., sticking to the ABM, the lower the level of analyte in the sample.

In a "sandwich assay", both an insolubilized ABM, and a labeled ABM are employed. The analyte is captured by the insolubilized ABM and is tagged by the labeled ABM, forming a ternary complex. The reagents may be added to the sample in either order, or simultaneously. The ABMs may be the same or different. The amount of labeled ABM in the ternary complex is directly proportional to the amount of analyte in the sample.

The two embodiments described above are both

heterogeneous assays. However, homogeneous assays are conceivable. The key is that the label be affected by whether or not the complex is formed.

Conjugation Methods

A label may be conjugated, directly or indirectly (e.g., through a labeled anti-ABM antibody), covalently (e.g., with SPDP) or noncovalently, to the ABM, to produce a diagnostic reagent. Similarly, the ABM may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent.

Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention.

The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

Biological Assays

A biological assay measures or detects a biological response of a biological entity to a substance.

The biological entity may be a whole organism, an isolated organ or tissue, freshly isolated cells, an immortalized cell line, or a subcellular component (such as a membrane; this term should not be construed as including an isolated receptor). The entity may be, or may be derived from, an organism which occurs in nature, or which is modified in some way. Modifications may be genetic (including radiation and chemical mutants, and genetic engineering) or somatic (e.g., surgical, chemical, etc.). In the case of a multicellular entity, the modifications may affect some or all cells. The entity need not be the target

organism, or a derivative thereof, if there is a reasonable correlation between bioassay activity in the assay entity and biological activity in the target organism.

The entity is placed in a particular environment, which may be more or less natural. For example, a culture medium may, but need not, contain serum or serum substitutes, and it may, but need not, include a support matrix of some kind, it may be still, or agitated. It may contain particular biological or chemical agents, or have particular physical parameters (e.g., temperature), that are intended to nourish or challenge the biological entity.

There must also be a detectable biological marker for the response. At the cellular level, the most common markers are cell survival and proliferation, cell behavior (clustering, motility), cell morphology (shape, color), and biochemical activity (overall DNA synthesis, overall protein synthesis, and specific metabolic activities, such as utilization of particular nutrients, e.g., consumption of oxygen, production of CO_2, production of organic acids, uptake or discharge of ions).

The direct signal produced by the biological marker may be transformed by a signal producing system into a different signal which is more observable, for example, a fluorescent or colorimetric signal.

The entity, environment, marker and signal producing system are chosen to achieve a clinically acceptable level of sensitivity, specificity and accuracy.

In some cases, the goal will be to identify substances which mediate the biological activity of a natural biological entity, and the assay is carried out directly with that entity. In other cases, the biological entity is used simply as a model of some more complex (or otherwise inconvenient to work with) biological entity. In that event, the model biological entity is used because activity in the model system is considered more predictive of activity in the ultimate natural biological entity than is simple binding activity in an in vitro system. The model entity is used instead of the ultimate entity because the

former is more expensive or slower to work with, or because ethical considerations forbid working with the ultimate entity yet.

The model entity may be naturally occurring, if the model entity usefully models the ultimate entity under some conditions. Or it may be non-naturally occurring, with modifications that increase its resemblance to the ultimate entity.

Transgenic animals, such as transgenic mice, rats, and rabbits, have been found useful as model systems.

In cell-based model assays, where the biological activity is mediated by binding to a receptor (target protein), the receptor may be functionally connected to a signal (biological marker) producing system, which may be endogenous or exogenous to the cell.
There are a number of techniques of doing this.

"Zero-Hybrid" Systems

In these systems, the binding of a peptide to the target protein results in a screenable or selectable phenotypic change, without resort to fusing the target protein (or a ligand binding moiety thereof) to an endogenous protein. It may be that the target protein is endogenous to the host cell, or is substantially identical to an endogenous receptor so that it can take advantage of the latter's native signal transduction pathway. Or sufficient elements of the signal transduction pathway normally associated with the target protein may be engineered into the cell so that the cell signals binding to the target protein.

"One-Hybrid" Systems

In these systems, a chimera receptor, a hybrid of the target protein and an endogenous receptor, is used. The chimeric receptor has the ligand binding characteristics of the target protein and the signal transduction characteristics of the endogenous receptor. Thus, the normal signal transduction pathway of the endogenous

receptor is subverted.

Preferably, the endogenous receptor is inactivated, or the conditions of the assay avoid activation of the endogenous receptor, to improve the signal-to-noise ratio.

See Fowlkes USP 5,789,184 for a yeast system.

Another type of "one-hybrid" system combines a peptide: DNA-binding domain fusion with an unfused target receptor that possesses an activation domain.

"Two-Hybrid" System

In a preferred embodiment, the cell-based assay is a two hybrid system. This term implies that the ligand is incorporated into a first hybrid protein, and the receptor into a second hybrid protein. The first hybrid also comprises component A of a signal generating system, and the second hybrid comprises component B of that system. Components A and B, by themselves, are insufficient to generate a signal. However, if the ligand binds the receptor, components A and B are brought into sufficiently close proximity so that they can cooperate to generate a signal.

Components A and B may naturally occur, or be substantially identical to moieties which naturally occur, as components of a single naturally occurring biomolecule, or they may naturally occur, or be substantially identical to moieties which naturally occur, as separate naturally occurring biomolecules which interact in nature.

Two-Hybrid System: Transcription Factor Type

In a preferred "two-hybrid" embodiment, one member of a peptide ligand:receptor binding pair is expressed as a fusion to a DNA-binding domain (DBD) from a transcription factor (this fusion protein is called the "bait"), and the other is expressed as a fusion to a transactivation domain (TAD) (this fusion protein is called the "fish", the "prey", or the "catch"). The transactivation domain should be complementary to the DNA-binding domain, i.e., it should interact with the latter so as to activate transcription of

a specially designed reporter gene that carries a binding site for the DNA-binding domain. Naturally, the two fusion proteins must likewise be complementary.

This complementarity may be achieved by use of the complementary and separable DNA-binding and transcriptional activator domains of a single transcriptional activator protein, or one may use complementary domains derived from different proteins. The domains may be identical to the native domains, or mutants thereof. The assay members may be fused directly to the DBD or TAD, or fused through an intermediated linker.

The target DNA operator may be the native operator sequence, or a mutant operator. Mutations in the operator may be coordinated with mutations in the DBD and the TAD. An example of a suitable transcription activation system is one comprising the DNA-binding domain from the bacterial repressor LexA and the activation domain from the yeast transcription factor Gal4, with the reporter gene operably linked to the LexA operator.

It is not necessary to employ the intact target receptor; just the ligand-binding moiety is sufficient.

The two fusion proteins may be expressed from the same or different vectors. Likewise, the activatable reporter gene may be expressed from the same vector as either fusion protein (or both proteins), or from a third vector.

Potential DNA-binding domains include Gal4, LexA, and mutant domains substantially identical to the above.

Potential activation domains include E. coli B42, Gal4 activation domain II, and HSV VP16, and mutant domains substantially identical to the above.

Potential operators include the native operators for the desired activation domain, and mutant domains substantially identical to the native operator.

The fusion proteins may comprise nuclear localization signals.

The assay system will include a signal producing system, too. The first element of this system is a reporter gene operably linked to an operator responsive to the DBD

and TAD of choice. The expression of this reporter gene will result, directly or indirectly, in a selectable or screenable phenotype (the signal). The signal producing system may include, besides the reporter gene, additional genetic or biochemical elements which cooperate in the production of the signal. Such an element could be, for example, a selective agent in the cell growth medium. There may be more than one signal producing system, and the system may include more than one reporter gene.

The sensitivity of the system may be adjusted by, e.g., use of competitive inhibitors of any step in the activation or signal production process, increasing or decreasing the number of operators, using a stronger or weaker DBD or TAD, etc.

When the signal is the death or survival of the cell in question, or proliferation or nonproliferation of the cell in question, the assay is said to be a selection. When the signal merely results in a detectable phenotype by which the signaling cell may be differentiated from the same cell in a nonsignaling state (either way being a living cell), the assay is a screen. However, the term "screening assay" may be used in a broader sense to include a selection. When the narrower sense is intended, we will use the term "nonselective screen".

Various screening and selection systems are discussed in Ladner, USP 5,198,346.

Screening and selection may be for or against the peptide: target protein or compound:target protein interaction.

Preferred assay cells are microbial (bacterial, yeast, algal, protozooal), invertebrate, vertebrate (esp. mammalian, particularly human). The best developed two-hybrid assays are yeast and mammalian systems.

Normally, two hybrid assays are used to determine whether a protein X and a protein Y interact, by virtue of their ability to reconstitute the interaction of the DBD and the TAD. However, augmented two-hybrid assays have been used to detect interactions that depend on a third, non-

protein ligand.

For more guidance on two-hybrid assays, see Brent and Finley, Jr., Ann. Rev. Genet., 31:663-704 (1997); Fremont-Racine, et al., Nature Genetics, 277-281 (16 July 1997); Allen, et al., TIBS, 511-16 (Dec. 1995); LeCrenier, et al., BioEssays, 20:1-6 (1998); Xu, et al., Proc. Nat. Acad. sci. (USA), 94:12473-8 (Nov. 1992); Esotak, et al., Mol. Cell. Biol., 15:5820-9 (1995); Yang, et al., Nucleic Acids Res., 23:1152-6 (1995); Bendixen, et al., Nucleic Acids Res., 22:1778-9 (1994); Fuller, et al., BioTechniques, 25:85-92 (July 1998); Cohen, et al., PNAS (USA) 95:14272-7 (1998); Kolonin and Finley, Jr., PNAS (USA) 95:14266-71 (1998). See also Vasavada, et al., PNAS (USA), 88:10686-90 (1991) (contingent replication assay), and Rehrauer, et al., J. Biol. Chem., 271:23865-73 91996) (LexA repressor cleavage assay).

Two-Hybrid Systems: reporter Enzyme type

In another embodiment, the components A and B reconstitute an enzyme which is not a transcription factor.

As in the last example, the effect of the reconstitution of the enzyme is a phenotypic change which may be a screenable change, a selectable change, or both.

In vivo Diagnostic Uses

Radio-labeled ABM may be administered to the human or animal subject. Administration is typically by injection, e.g., intravenous or arterial or other means of administration in a quantity sufficient to permit subsequent dynamic and/or static imaging using suitable radio-detecting devices. The dosage is the smallest amount capable of providing a diagnostically effective image, and may be determined by means conventional in the art, using known radio-imaging agents as a guide.

Typically, the imaging is carried out on the whole body of the subject, or on that portion of the body or organ relevant to the condition or disease under study. The

amount of radio-labeled ABM accumulated at a given point in time in relevant target organs can then be quantified.

A particularly suitable radio-detecting device is a scintillation camera, such as a gamma camera. A scintillation camera is a stationary device that can be used to image distribution of radio-labeled ABM. The detection device in the camera senses the radioactive decay, the distribution of which can be recorded. Data produced by the imaging system can be digitized. The digitized information can be analyzed over time discontinuously or continuously. The digitized data can be processed to produce images, called frames, of the pattern of uptake of the radio-labelled ABM in the target organ at a discrete point in time. In most continuous (dynamic) studies, quantitative data is obtained by observing changes in distributions of radioactive decay in target organs over time. In other words, a time-activity analysis of the data will illustrate uptake through clearance of the radio-labeled binding protein by the target organs with time.

Various factors should be taken into consideration in selecting an appropriate radioisotope. The radioisotope must be selected with a view to obtaining good quality resolution upon imaging, should be safe for diagnostic use in humans and animals, and should preferably have a short physical half-life so as to decrease the amount of radiation received by the body. The radioisotope used should preferably be pharmacologically inert, and, in the quantities administered, should not have any substantial physiological effect.

The ABM may be radio-labeled with different isotopes of iodine, for example ^{123}I, ^{125}I, or ^{131}I (see for example, U.S. Patent 4,609,725). The extent of radio-labeling must, however be monitored, since it will affect the calculations made based on the imaging results (i.e. a diiodinated ABM will result in twice the radiation count of a similar monoiodinated ABM over the same time frame).

In applications to human subjects, it may be desirable to use radioisotopes other than ^{125}I for labeling in order to

decrease the total dosimetry exposure of the human body and to optimize the detectability of the labeled molecule (though this radioisotope can be used if circumstances require). Ready availability for clinical use is also a factor. Accordingly, for human applications, preferred radio-labels are for example, ^{99m}Tc, ^{67}Ga, ^{68}Ga, ^{90}Y, ^{111}In, ^{113m}In, ^{123}I, ^{186}Re, ^{188}Re or ^{211}At.

The radio-labelled ABM may be prepared by various methods. These include radio-halogenation by the chloramine - T method or the lactoperoxidase method and subsequent purification by HPLC (high pressure liquid chromatography), for example as described by J. Gutkowska et al in "Endocrinology and Metabolism Clinics of America: (1987) 16 (1):183. Other known methods of radio-labeling can be used, such as IODOBEADS™.

There are a number of different methods of delivering the radio-labeled ABM to the end-user. It may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption of an ABM, such as an antibody, which is a protein.

EXAMPLES

Example 1

Animal Models and Methods

Animal Models Three week-old male C57Bl/6 mice were placed on either a normal diet (PMI Nutrition Intenational, Inc., Brentwood, MO, Prolab RMH3000) or a high-fat diet (BioServe, Frenchtown, NJ, #F1850) for 8 weeks. High-Fat fed mice were chosen which qualified as hyperinsulinemic (but non-diabetic) or as diabetic, per criteria set forth previously. A mouse fed the normal diet and demonstrating normal weight gain, normal fasting plasma insulin levels and normal fasting blood glucose levels was chosen as the Control animal. Control, Hyperinsulinemic and Type II diabetic mice were sacrificed at 11 weeks of age (8 weeks on the feeding protocol) and total liver RNA were isolated. The mice chosen were:

Mouse #	Weight (g)	Fasting Plasma Insulin (ng/ml)	Fasting Plasma Glucose (mg/dl)
L-9 (Control)	28.7	0.62	135
H-34 (HI)	32.2	2.11	162
H-43 (D)	38.6	2.94	239

Fasting Blood Glucose Levels. Blood glucose levels was measured from a drop of blood taken from the tip of the tail of fasted (6 hr) mice using a Lifescan Genuine One Touch glucometer. All measurements occurred between 3:00 p.m. and 5:00 p.m.

Plasma insulin measurements. Blood was collected from the tail of fasted (6hr) mice into a heparinized capillary tube and stored on ice. All collections occurred between 3:00 p.m. and 5:00 p.m. Plasma was separated from red blood cells by centrifugation for 10 minutes at 8000 x g and then stored at -20°C. Insulin concentrations were determined

using the Rat Insulin ELISA kit and rat insulin standards (ALPCO) essentially as instructed by the manufacturer. Values were adjusted by a factor of 1.23 as determined by the manufacturer to correct for the species difference in cross-reactivity with the antibody.

RNA isolation Total RNA was isolated from livers using the RNA STAT-60 Total RNA/mRNA Isolation Reagent according to the manufacturer's instructions (Tel-Test, Friendswood, TX).

cDNA synthesis cDNA was synthesized using 1 µg of total RNA from L-9, H-34 and H-43 mice using the SMART PCR cDNA Synthesis Kit according to the manufacturer's instructions (CLONTECH, Palo Alto, CA).

Generation of cDNA subtraction libraries Forward- and reverse-subtracted cDNA libraries were generated using the PCR-Select cDNA Subtraction Kit (CLONTECH, Palo Alto, CA) and the L-9, H-34 and H43 samples. Library (A) included clones down-regulated in control mice compared to hyperinsulinemic (HI) mice, Library (Z) included clones up-regulated in control mice compared to hyperinsulinemic mice; Library (B) included clones down-regulated in Type-II diabetic mice compared to hyperinsulinemic mice; and Library (Y) included clones up-regulated in Type-II diabetic mice compared to hyperinsulinemic mice.

Isolation of individual clones After generating the cDNA subtraction libraries, the PCR product ends were made blunt by treatment with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and subcloned into a bacterial plasmid vector using the Zero Blunt TOPO PCR Cloning Kit as instructed by the manufacturer (Invitrogen Corp., Carlsbad, CA). Individual clones were obtained by plating on selective media.

Screening by differential hybridization cDNA arrays of clones from the forward and reverse subtracted libraries

were screened with probes made from each library using the PCR-Select Differential Screening Kit according to the manufacturer's instructions (CLONTECH, Palo Alto, CA).

Nucleotide sequence determination Plasmid DNA from bacterial colonies carrying the differentially expressed cDNA inserts was isolated using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions (Qiagen Inc., Santa Clarita, CA). Nucleotide sequences were determined by use of the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with electrophoresis on the ABI PRISM 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA.). Nucleotide sequences and predicted amino acid sequences were compared to public domain databases using the Blast 2.0 program (National Center for Biotechnology Information, National Institutes of Health).

Northern analysis Positive clones, identified by the differential hybridization screen, were used as probes in Northern hybridization analyses to confirm their differential expression. Total RNA isolated from Control, hyperinsulinemic and Type-II Diabetic mice was resolved by agarose gel electrophoresis through a 1% agarose, 1 % formaldehyde denaturing gel, transferred to positively charged nylon membrane, hybridized to a probe labeled with [32P] dCTP that was generated from the cDNA insert using the Random Primed DNA Labeling Kit (Roche, Palo Alto, CA).

Database Searches Nucleotide sequences and predicted amino acid sequences were compared to public domain databases using the Blast 2.0 program (National Center for Biotechnology Information, National Institutes of Health). Nucleotide sequences were displayed using ABI prism Edit View 1.0.1 (PE Applied Biosystems, Foster City, CA).

Nucleotide database searches were conducted with the then current version of BLASTN 2.0.12, see Altschul, et al., "Gapped BLAST and PSI-BLAST: a new generation of protein

database search programs", Nucleic Acids Res., 25:3389-3402 (1997). Searches employed the default parameters, unless otherwise stated.

For blastN searches, the default was the blastN matrix (1,-3), with gap penalties of 5 for existence and 2 for extension.

Protein database searches were conducted with the then-current version of BLAST X, see Altschul et al. (1997), supra. Searches employed the default parameters, unless otherwise stated. The scoring matrix was BLOSUM62, with gap costs of 11 for existence and 1 for extension. The standard low complexity filter was used.

"ref" indicates that NCBI's RefSeq is the source database. The identifier that follows is a RefSeq accession number, not a GenBank accession number. "RefSeq sequences are derived from GenBank and provide non-redundant curated data representing our current knowledge of known genes. Some records include additional sequence information that was never submitted to an archival database but is available in the literature. A small number of sequences are provided through collaboration; the underlying primary sequence data is available in GenBank, but may not be available in any one GenBank record. RefSeq sequences are not submitted primary sequences. RefSeq records are owned by NCBI and therefore can be updated as needed to maintain current annotation or to incorporate additional sequence information." See also http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html

It will be appreciated by those in the art that the exact results of a database search will change from day to day, as new sequences are added. Also, if you query with a longer version of the original sequence, the results will change. The results given here were obtained at one time and no guarantee is made that the exact same hits would be obtained in a search on the filing date. However, if an alignment between a particular query sequence and a particular database sequence is discussed, that alignment should not change (if the parameters and sequences remain unchanged).

CHARACTERIZATION OF CLONES

Clone Z41 Insert size: 946 bp query sequence (SEQ ID NO: 1)

Nucleotide database search

Blast N:

Clone Z41 is apparently a partial cDNA of a gene encoding the Mus musculus cytochrome P450 3a11 (Cyp3a11) protein. The highest scoring DNA alignment (1049 bits, E value 0.0) was of bases 82-699 of clone Z41 with bases 744-1357 of Cyp3a11 (NM_007818) mRNA, length=1690. The percentage identity was 97% (601/619) with 6 gaps.

The highest human matches in the main database were members of the cytochrome P450 subfamily 3. Note that these proteins are functionally related. The cytochrome P450 proteins are monooxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.

The highest human matches were:

NM_017460: cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4 (CYP3A4), transcript variant 1, complete sequence. This protein localizes to the endoplasmic reticulum and its expression is induced by glucocorticoids and some pharmacological agents. This enzyme is involved in the metabolism of approximately half the drugs which are are used today, including acetaminophen codeine, cyclosporin A, diazepam and erythromycin.

NM_000776: cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 3 (CYP3A3), complete sequence.

X12387: mRNA for cytochrome P-450 (cyp3 locus, complete sequence.

AF182273: cytochrome P450-3A4 (CYP3A4) mRNA, complete sequence.

M18907: P450 mRNA encoding nifedipine oxidase, complete sequence.

M13785: Liver glucocorticoid-inducible cytochrome P-450 (HLp) mRNA, complete sequence.

BC033862/NM_000777: cytochrome P450, subfamily IIIA(CYP3A5)(niphedipine oxidase), polypeptide 5, complete

sequence.
J04814: cytochrome P450 PCN3 mRNA, complete sequence.

Protein database search
Blast X:
The best score in the main database was with Mus musculus-cytochrome P450, steroid inducible 3a11, NP_031844 (score 315 bits, e value 4e-86).
Again, the highest human matches in the main database were members of the cytochrome P450 subfamily 3 as follows:
NP_000767: cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 3
NP_000768: cytochrome P450, subfamily IIIA, polypeptide 5
AAA35747: cytochrome P450 nifedipine oxidase
NP_059488: cytochrome P450, subfamily IIIA, polypeptide 4; nifedipine oxidase; P450-III, steroid inducible; glucocorticoid-inducible P450; cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 3
NP_000756: cytochrome P450, subfamily IIIA, polypeptide 7
NP_073731: cytochrome P450, family 3, subfamily A polypeptide 43 isoform 1.
NP_476436: cytochrome P450, family 3, subfamily A,polypeptide 43 isoform 2.
NP_476437: cytochrome P450 polypeptide 43; cytochrome P450, subfamily IIIA, polypeptide 43

Clone Z74 Insert size: 916 bp query sequence (SEQ ID NO: 2)

Nucleotide database search
Blast N:
Clone Z74 is apparently a partial cDNA of a gene encoding the Mus musculus synovial sarcoma translocation, mRNA. The highest scoring DNA alignment (1035 bits, E value 0.0) was bases 53-605 of clone Z74 with bases 2633-2081 of synovial sarcoma translocation, Chromosome 18 (Ss18) (NM_009280), mRNA, length=3107. The percentage identity was 98% (548/554) with 2 gaps. (Note that since the orientation of

the cDNA inserts in the cloning vector was not known, "plus" was assigned arbitrarily for the purpose of the Blast alignment. So, since the match is to the minus strand of a known DNA, we assume that the strand labeled "plus" was actually the minus strand of NM_009280)

The corresponding protein ID for NM_009280 is NP_033306: synovial sarcoma translocation, Chromosome 18; synovial sarcoma translocated to X chromosome. This protein represents the mouse homolog of SYT, a gene implicated in the development of human synovial sarcomas as described in de Bruijn,D.R. et al., Oncogene 13 (3), 643-648 (1996)

The highest human match in the main database was with synovial sarcoma translocation, chromosome 18 (SS18) (AF244972), mRNA, complete sequence. (Score = 79.8 bits (40), Expect = 4e-12, Identities = 81/92 (88%), Gaps = 2/92

Protein database search

Blast X: No significant similarity found. This is expected; the coding sequence of NM_009280 is from bases 180 to 1436. The sequence isolated as clone Z74 is therefore within the 3' untranslated region of NM_009280.

Human Blast P:

Blast P of NP_0033306 indicated significant homology to:
AAG31034: SYT/SSX4 fusion protein
AAM00188: SYT protein
AAK21314: SYT variant 1
NP_005628: Synovial sarcoma, translocated to X chromosome

Clone Y92

Insert size: 832 bp query sequence (SEQ ID NO: 8)

Nucleotide database search

Blast N:

Clone Y92 is apparently a partial cDNA of a gene encoding the Mus musculus cytochrome P450, 4a14 (Cyp4a14), mRNA. The highest scoring DNA alignment (1267 bits, E value 0.0) was bases 41-723 of clone Y92 with bases 731-1412 of (Cyp4a14; NM_007822), mRNA, length=2547. The percentage identity was 98% (675/685) with 5 gaps.

The highest human matches in the main database were members of the cytochrome P450 subfamily 4. Note that these proteins are functionally related. The cytochrome P450 proteins are monooxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.

The highest human matches were:

NM_000778: cytochrome P450, family 4, subfamily A, polypeptide 11 (CYP4A11), mRNA, complete sequence.

S67581: CYP4A11=fatty acid omega-hydroxylase [human, kidney, mRNA Mutant, complete sequence.

S67580: CYP4A11=fatty acid omega-hydroxylase [human, kidney, mRNA, complete sequence.

L04751: cytochrome p-450 4A (CYP4A) mRNA, complete sequence.

D13705: mRNA for fatty acids omega-hydroxylase (cytochrome P-450HKV), complete sequence.

Protein database search

Blast X:

The best score in the main database was with Mus musculus-cytochrome P450 4a14, NP_031848 (score 392 bits, e value e-109).

Again, the highest human matches in the main database were members of the cytochrome P450 subfamily 4 as follows:

I65981: fatty acid omega-hydroxylase; cytochrome P450 4A11 (Score 303, e value 3e-82)

NP_000769: cytochrome P450, subfamily IVA, polypeptide 11; fatty acid omega-hydroxylase; P450HL-omega; alkane-1 monooxygenase; lauric acid omega-hydroxylase

BAA02864: fatty acid omega-hydroxylase

O4HUB1: cytochrome P450 4B1

NP_000770: cytochrome P450, subfamily IVB, polypeptide 1; cytochrome P450, subfamily IVB, member 1; microsomal monooxygenase

Clone Z19

Insert size: 953 bp query sequence (SEQ ID NO: 11)

Nucleotide database search

Blast N:

Clone Z19 is apparently a partial cDNA of a gene encoding the Mus musculus RIKEN cDNA 2810007J24, mRNA. The highest scoring DNA alignment (611 bits, E value e-173) was bases 100-444 of clone Z19 with bases 1517-1174 of RIKEN cDNA 2810007J24 (XM_133188) mRNA, length = 2134bp. The percentage identity was 97% (335/345) with 1 gap (Note that since the orientation of the cDNA inserts in the cloning vector was not known, "plus" was assigned arbitrarily for the purpose of the Blast alignment. So, since the match is to the minus strand of a known DNA, we assume that the strand labeled "plus" was actually the minus strand of XM_133188). The region of XM_133188 between bases 385 to 1086 has been identified as a potential Sulfotransferase region. Therefore, XM_133188 may encode a Sulfotransferase protein.

Human Blast N: No significant similarity found.

Protein database search

Blast X: No significant similarity found. This is expected; the coding sequence of XM_133188 is from bases 277 to 1125. The sequence isolated as clone Z19 is therefore within the 3' untranslated region of XM_133188. The mouse protein corresponding to XM_133188 is XP_133188.

Clone A17

Insert size: 864 bp query sequence (SEQ ID NO: 3)

Nucleotide database search

Blast N

Clone A17 is apparently a partial cDNA of a gene encoding the Mus musculus kallistatin-related protein, mRNA. The highest scoring DNA alignment (698 bits, E value 0.0) was bases 75-757 of clone A17 with bases 504-1160 of Kallistatin-related protein (AF453874) gene, length=9218. The percentage identity was 92% (634/687) with 34 gaps.

Human Blast N: No significant similarity found.

Protein database search

Blast X: No significant similarity found. This is expected; the coding sequence of AF453874 is from bases 2127 to 2513. The sequence isolated as clone A17 is therefore within the 5' untranslated region of AF453874.

Blast X of AF453874 indicated significant homology to: human serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4; protease inhibitor 4 (kallistatin) (NP_006206). Score =116 bits, Expect = 1e-23. The corresponding human gene encodes the serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4 (SERPINA4), mRNA (NM_006215).

There was also significant homology (8e-25 to 1e-24) to several "unnamed proteins" that may represent the human kallistatin-related protein.

Clone A53

Insert size: 800 bp query sequence (SEQ ID NO: 10)

Nucleotide database search

Blast N

Clone A53 is apparently a partial cDNA of a gene encoding the Mus musculus adult male liver tumor cDNA, RIKEN clone: C730029H04 product, mRNA. The highest scoring DNA alignment (1255 bits, E value 0.0) was bases 55-710 of clone A53 with bases 2083-2738 of RIKEN clone: C730029H04 (AK050237) cDNA, Length = 2738. The percentage identity was 99%

(653/657) with 2 gaps.

Human Blast N: No significant similarity found.

Protein database search

Blast X: No significant similarity found. This is expected; AK050237 contains 5 open reading frames (ORFs) located between bases 546 and 1417, the longest being 246 bases (82 aa). The sequences contained within clone A53 are located outside of this region (bases 2083-2738). Therefore, clone A53 may represent a portion of either the 5' UTR or 3'UTR of AK050237.

Blast X of AK050237 indicated significant homology to: Human one cut domain, family member 1; hepatocyte nuclear factor 6, alpha (NP_004489) Score=175 bits, Expect = 5e-42 Identities = 89/113 (78%), Positives = 93/113 (82%). The human corresponding gene encodes the one cut domain, family member 1 (ONECUT1), mRNA (NM_004498).

Clone A104

Insert size: 751 bp query sequence (SEQ ID NO: 12)

Nucleotide database search

Blast N

Clone A104 is apparently a partial cDNA of a gene encoding the Mus musculus H2A histone family, member Y (H2afy),mRNA. The highest scoring DNA alignment (894 bits, E value 0.0) was bases 32-452 of clone A104 with bases 613-1083 of H2A histone family, member Y (H2afy) (XM_127380), mRNA, Length = 1756. The percentage identity was 98% (465/471).
The highest human matches in the main database were:
NM_004893: Homo sapiens H2A histone family, member Y (H2AFY), transcript variant 2, complete sequence.
BC013331: Homo sapiens, clone MGC:13692 IMAGE:4077577, complete sequence

AF054174: Homo sapiens histone macroH2A1.2, complete sequence

Protein database search

Blast X:

There were 3 equally significant scores in the main database for mouse proteins:

XP_127380: H2A histone family, member Y

AF171080: histone macroH2A1.2 variant

BAB68541: MacroH2A1.2

The highest human matches in the main database were members of the histone family as follows:

NP_004884: H2A histone family, member Y isoform 2; histone macroH2A1.2; histone macroH2A1.1

AAC39908: histone macroH2A1.2

NP_613258: H2A histone family, member Y isoform 3; histone macroH2A1.2; histone macroH2A1.1

H2AY_HUMAN: Core histone macro-H2A.1 (Histone macroH2A1) (mH2A1) (H2A.y) (H2A/y)

NP_613075: H2A histone family, member Y isoform 1; histone macroH2A1.2; histone macroH2A1.1

As well as: AAH13331: Unknown (protein for MGC:13692)

Clone B8

Insert size: 829 bp query sequence (SEQ ID NO: 4)

Nucleotide database search

Blast N

Clone B8 is apparently a partial cDNA of a gene encoding the Mus musculus liver-specific uridine phosphorylase, mRNA. The scoring DNA alignment (649 bits, E value 0.0) was bases 334-691 of clone B8 with bases 356-1 of liver-specific uridine phosphorylase (AY152393), mRNA, Length = 1627. The percentage identity was 98% (352/358) with 2 gaps. (Note that since the orientation of the cDNA inserts in the cloning vector was not known, "plus" was assigned

arbitrarily for the purpose of the Blast alignment. So, since the match is to the minus strand of a known DNA, we assume that the strand labeled "plus" was actually the minus strand of AY152393).

The three possible corresponding human genes are:
NM_173355: Liver-specific uridine phosphorylase (LOC151531)
XM_087230: Similar to Uridine phosphorylase (UDRPase) (LOC151531)
XM_087230: Similar to Uridine phosphorylase (UDRPase) (LOC151531)

Protein database search

Blast X:

The corresponding Mus musculus proteins are:
NP_083968: liver-specific uridine phosphorylase
AAO05705: liver-specific uridine phosphorylase

The corresponding human proteins are either liver-specific uridine phosphorylase or a protein similar to uridine phosphorylase as follows:
NP_775491: Liver-specific uridine phosphorylase
XP_087230: Similar to Uridine phosphorylase (UDRPase)
AAH33529: Similar to uridine phosphorylase
AAD12227: Similar to uridine phosphorylase; similar to Q16831 (PID:g2494059)

Clone B39

Insert size: 851 bp query sequence (SEQ ID NO: 5)

Nucleotide database search

Blast N

Clone B39 is apparently a partial cDNA of a gene encoding the Mus musculus TRAM1, mRNA. The scoring DNA alignment (442 bits, E value -121) was bases 69-361 of clone B39 with

bases 1819-1526 of TRAM1 (AY029764), mRNA, Length = 2720. The percentage identity was 93% (352/358) with 3 gaps. (Note that since the orientation of the cDNA inserts in the cloning vector was not known, "plus" was assigned arbitrarily for the purpose of the Blast alignment. So, since the match is to the minus strand of a known DNA, we assume that the strand labeled "plus" was actually the minus strand of AY029764).

It is possible that B39 may be a partial cDNA of the following three other Mus musculus genes:

BC012401: Clone MGC:11724 IMAGE:3967323, mRNA Length = 2819

AK088814: 2 days neonate thymus thymic cells cDNA, RIKEN clone:E430026I15 product:TRAM1 (UNKNOWN) (PROTEIN FOR MGC:11724), Length = 2889

AK028304: 17 days embryo head cDNA, RIKEN clone:3322402I02 product:TRAM1 (UNKNOWN) (PROTEIN FOR MGC:11724), Length = 2820

Human Blast N:

The only match in the Human database was to the MDM2 gene, intron 9 and exon 10(AF144029/F144014S16), partial sequence Length = 294, Score = 333 bits, Expect = 3e-88

Protein database search

Blast X: No significant similarity found. This is expected; the coding sequence AY029764 is from bases 36 to 1160. The sequence isolated as clone B39 is therefore within the 3' untranslated region of AY029764.

Blast X of AY029764 indicates that the corresponding human protein is apparently the human translocating chain-associating membrane protein; translocating chain-associating membrane protein (TRAM) (NP_055109), Score = 662 bits (1708), Expect = 0.0, or a protein similar to TRAM. The corresponding human gene encodes the translocating chain-associating membrane protein (TRAM), mRNA (NM_014294).

The corresponding human proteins may also be the unknown protein for MGC:33851(AAH37738); the hypothetical

protein MGC26568 (NP_689615); the unnamed protein product (BAC11091) or the TRAM-like protein KIAA0057 (NP_036420)

Clone Y68

Insert size: 966 bp query sequence (SEQ ID NO: 6)

Nucleotide database search

Blast N

Clone Y68 is apparently a partial cDNA of a gene encoding either the Mus musculus, integral membrane protein 2B and or the E25B protein, mRNA. The scoring DNA alignment (1142 bits, E value 0.0) was bases 71-722 of clone Y68 with bases 550-1202 of integral membrane protein 2B (AK076139), mRNA, Length = 1783. The percentage identity was 96% (631/653) with 1 gap.

The scoring DNA alignment (1128 bits, E value 0.0) was bases 71-722 of clone Y68 with bases 561-1214 of E25B protein (AB030203), mRNA, Length = 1622. The percentage identity was 96% (631/653) with 2 gaps.

Human Blast N:

The highest human matches in the main database were to integral membrane protein 2B or to genes encoding proteins similar to integral membrane protein 2B.

The highest human matches were:

NM_021999: integral membrane protein 2B (ITM2B)

BC016148: Similar to integral membrane protein 2B, clone MGC:10219 IMAGE:3912066

BC000554: Similar to integral membrane protein 2B, cloneMGC:1034 IMAGE:3163436

AF152462: sapiens transmembrane protein BRI (BRI)

AF092128: putative transmembrane protein E3-16

Protein database search

Blast X:

The corresponding Mus musculus proteins are integral membrane protein 2B (AAH21786) and/or E25B protein (AAC63851).

Human Blast X:

The corresponding human proteins may be:
NP_068839: Integral membrane protein 2B
AAH16148: Similar to integral membrane protein 2B
AAH00554: Similar to integral membrane protein 2B
AAD40370 : putative transmembrane protein E3-16
AF152462: transmembrane protein BRI
BAA91210: unnamed protein product

Clone Y89

Insert size: 842 bp query sequence (SEQ ID NO: 9)

Nucleotide database search

Blast N

Clone Y89 is apparently a partial cDNA of a gene encoding the Mus musculus, vitronectin, clone MGC:21423 IMAGE:4500844 mRNA. The scoring DNA alignment (462 bits, E value e-128) was bases 33-269 of clone Y89 with bases 1509-1745 of vitronectin (BC018521), mRNA, Length = 1783. The percentage identity was 99% (236/237).

The corresponding human gene is:
NM_000638: Vitronectin (serum spreading factor, somatomedin B, complement S-protein) (VTN), Score = 143 bits, Expect = 5e-31, Identities = 123/140 (87%)

Protein database search

Blast X:

The corresponding Mus musculus protein is Vitronectin (AAH18521)
The corresponding Human protein is: NP_000629: Vitronectin; serum spreading factor; somatomedin B; complement S-protein

Clone Y91

Insert size: 8806bp query sequence (SEQ ID NO: 7)

Nucleotide database search

Blast N

Clone Y91 is apparently a partial cDNA of a gene encoding the Mus musculus, SPI-2 mRNA. The scoring DNA alignment (1090 bits, E value 0.0) was bases 76-788 of clone Y91 with bases 1594-892 of SPI-2 (X56786), mRNA, Length = 1650. The percentage identity was 95% (683/714) with 12 gaps (Note that since the orientation of the cDNA inserts in the cloning vector was not known, "plus" was assigned arbitrarily for the purpose of the Blast alignment. So, since the match is to the minus strand of a known DNA, we assume that the strand labeled "plus" was actually the minus strand of X56786).

Human Blast N

There were no significant human matches. The highest scoring human match was:

BC034554: Homo sapiens, serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3, clone MGC:18107 IMAGE:4152390, mRNA, Length = 1584, Score = 56.0 bits (28), Expect = 9e-05, Identities = 55/64 (85%)

Protein database search

Blast X:

The corresponding Mus musculus protein is contraspin (CAA40106).

Human Blast X

There were no significant human matches. The highest scoring human match was:

alpha1-antichymotrypsin (CAA48671), Score = 72.0 bits (175), Expect = 2e-11.

NOTE: contrapsin is a member of the serpin superfamily and inhibits trypsin much more strongly than alpha1-antiproteinase. Mouse and rat contrapsins, however, have similarity in sequence to human alpha1- antichymotrypsin (Yoshida et al., 2001).

Citation of documents herein is not intended as an admission that any of the documents cited herein is pertinent prior art, or an admission that the cited documents is considered material to the patentability of any of the claims of the present application. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

The appended claims are to be treated as a non-limiting recitation of preferred embodiments.

In addition to those set forth elsewhere, the following references are hereby incorporated by reference, in their most recent editions as of the time of filing of this application: Kay, Phage Display of Peptides and Proteins: A Laboratory Manual; the John Wiley and Sons Current Protocols series, including Ausubel, Current Protocols in Molecular Biology; Coligan, Current Protocols in Protein Science; Coligan, Current Protocols in Immunology; Current Protocols in Human Genetics; Current Protocols in Cytometry; Current Protocols in Pharmacology; Current Protocols in Neuroscience; Current Protocols in Cell Biology; Current Protocols in Toxicology; Current Protocols in Field Analytical Chemistry; Current Protocols in Nucleic Acid Chemistry; and Current Protocols in Human Genetics; and the following Cold Spring Harbor Laboratory publications: Sambrook, Molecular Cloning: A Laboratory Manual; Harlow, Antibodies: A Laboratory Manual; Manipulating the Mouse Embryo: A Laboratory Manual; Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual; Drosophila Protocols; Imaging Neurons: A Laboratory Manual; Early Development of Xenopus laevis: A Laboratory Manual; Using Antibodies: A Laboratory Manual; At the Bench: A Laboratory Navigator; Cells: A Laboratory Manual; Methods in Yeast Genetics: A Laboratory Course Manual; Discovering Neurons: The Experimental Basis of Neuroscience; Genome Analysis: A Laboratory Manual Series ; Laboratory DNA Science;

Strategies for Protein Purification and Characterization: A Laboratory Course Manual; Genetic Analysis of Pathogenic Bacteria: A Laboratory Manual; PCR Primer: A Laboratory Manual; Methods in Plant Molecular Biology: A Laboratory Course Manual ; Manipulating the Mouse Embryo: A Laboratory Manual; Molecular Probes of the Nervous System; Experiments with Fission Yeast: A Laboratory Course Manual; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria; DNA Science: A First Course in Recombinant DNA Technology; Methods in Yeast Genetics: A Laboratory Course Manual; Molecular Biology of Plants: A Laboratory Course Manual.

All references cited herein, including journal articles or abstracts, published, corresponding, prior or otherwise related U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for

the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

Any description of a class or range as being useful or preferred in the practice of the invention shall be deemed a description of any subclass (e.g., a disclosed class with one or more disclosed members omitted) or subrange contained therein, as well as a separate description of each individual member or value in said class or range.

The description of preferred embodiments individually shall be deemed a description of any possible combination of such preferred embodiments, except for combinations which are impossible (e.g, mutually exclusive choices for an element of the invention) or which are expressly excluded by this specification.

If an embodiment of this invention is disclosed in the prior art, the description of the invention shall be deemed to include the invention as herein disclosed with such embodiment excised.

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Table 1

Clone Z41

Clone Sequence: (1-946) *(SEQ ID NO:1)*

attttggggccactcggatttntagtaacggccgcagtgtgctggaattcgcccttagcg
tggtcgcggccgcccgggcaggtacttttttccattcctgacaccagtatatgagatgtta
aatacctgcatgttcccaaaggattcaatagaatttttcaaaaaatttgtggacagaatg
aaggaaagccgcctggattctaagcagaagcaccgagtggattttcttcagctgatgatg
aattctcataataattccaaagacaaagtctctcataaagccctttctgacatggagatc
acagcccagtcaattatctttatttttgctgggtatgaaaccaccagtagcacactttcc
ttcaccctgcattccttggccactcaccctgatatccagaaaaaactgcaggatgagatc
gatgaggctctgcccaacaaggcacctcccacgtatgatactgtgatggagatggaatac
ctggatatggtgcttaatgaaaccctcagattatatcccattgctaatagacttgggaga
gtctgtaagaaagatgtggaactcntgggtgtgtatattccccaaaggggtcaacagtga
tgatccatcttatgctcttcaccattgaccccnagcactggtcnagagcctgaanaattn
caaccttgaaaggttcagnnaggagaacaagggcagcatgnaccttnctatatctcngct
ttngggaatggnccnaggactncntngcatganggcttgntccatgatnnaantgntgta
ctaaatnnnccaanttntcttccncntggaaggacncngnccttgnaataagcaaaagnc
ttttngnccaaaaccattgttnagtggccccganctcntnnggnctagccctnagatntn
nnttnaagggnntnnaaccgcttnttactnagaagnnnaggggnan

Table 2

Clone Z74

Clone Sequence: (1-916) *(SEQ ID NO:2)*

gntccnagtnngcgcagagtgctggattcgccttagcgtggtcgcggccgaggtacatgt
tttcttgccccatggcatatccccaaatttatgctaaaataatcagtctcaaataatgat
taagtatgtcgtgcttaaaaacaagaaaccgtaaaatgtcacctctcggctttcagtgcg
aatgagaaatggccatcaaggcattttcctcgcgcattaactaagatgaagagaggaaca
cgaaggcgccctttgcttgtgcccagttaagaaagttaaagcacaagcgtgtgaagtctc
gtggactcctccctgctccatgaaccaatcatggtaatgtaaaaaacagtaaggtgagac
caatgctgcctcctctctgacgtggtgagagctccctgattacaagacaaggtaaaaaca
tcccgcttcctcctttctgttattgctgttttaagagaggcttccctcctttacacaa
acctgccaaagtccacagatgcaacataattattgccttacgtttgaaaacagtattata
aaccatatttacaaaaaggttttaatgcacaaaactggaaggctttttaaaaatgtcttt
gtactgcccgggcggccgctcgaagggcgaattctgcagatatccatcacactggcggcc
gctcgagcatgcatctagaagggccaattcgcctatagtgagtngtttncaattcactgg
ccgtcgttttcnacgtcgtgacgggaaaccctggcgntaccaanttatnccttgcagcnc
atccccttttcnccnctggcgtatacgaaaagcccncgancccttccaaagtggcactanc
gtaggggttangtcccttaaaaaancgttcnttttttgannnaagnttttnnccgggngga
ggaccnncagcatcnn

Table 3

Clone A17

Clone Sequence: (1-864) *(SEQ ID NO:3)*

gcagcctgancnngnanctncngngagaggaatccccttgcgtggcngcggcgaggnca
tcgtatcacacaatcagacacacacacatgnaaaagtaagtaaattatttaagtgaggaa
atagcntggggatcntagaggtgaagcntcagctagcagtttgcantaagatgcactgag
gnttcagagcatcattncatagtgcaagaggagaagaaagaaagatagacctgtgntttg
tgaccctcttaaggttatttgaattttagactgcagggcccatgcatagagttttgttag
aaatgcattgccattgaccacagattgcatcccaantgtccagggctggagataanacaa
tggcaaaagagactggcattgcaatgcaagggatgacatccntcatggcccatttgttgg
aagtttttcatcatcatggtcatgagggagagacaatgaagacaaaagcaataatccatt
gtgggtgtgcagggagggcaggggaggagtgatccaaagagaaactctcaggatatgaca
tgggaaggggtgcacatagaaggcatggagatatagtcacaggggaaccnaacagtgat
aggaccaatggatgggacaagggtagagtcagtgtagaaaatctacaggctttcttgggc
aggtcangttcattgtgggaaggggaattggtgcncctattgtaagggtagggtgagacc
attcattgcaaatnatcctttctggatggggcagtggnaaaagacactcttcttccctcn
ttggagaaacccaggaagtgangaggttgaatgtanttgacagccaggana acccaantn
ctcccatccncctggctgcccgag

Table 4

Clone B8

Clone Sequence: (1-829) *(SEQ ID NO:4)*

ggnanttcggnttttagtaacggccgccagtgtgctggaattcgcccttcgagcggccg
cccgggcaggtacgttctcagtgcccacgtatgtagacttccacatttcatatcagataa
cactgctctggcttgcatttgagcccacatctctgttgcaggattcagaaaaaaaaaaat
gaaactgacttantttttcttggagggaaaaagatacttagtggtgcctggctgatgtgg
gcagcattgggcagcagatccaaagaaggctagcaccggccttcatgggggctgtctgga
gaaattcctgaggttgctgagtgggtgtccctcctttacatccccaaacattgctggtag
gttgtgtgttttggttcctaaatccaagtgatagagaatgtcttcgtccatgccttccag
gtatggattcttaacatacacagaccgttttctctcngatgtattcttgtcaggtctcat
ggatctattagaagcaggcaaaatggaagccatactctgcggtgggcaaatagcaggaaa
aagcaatattagaggctgctgttaaattacagagctaatcacaatgatgcagcttcaagt
cagaataccaccttcccttcaaacttaggtttccttcttgaaatttcctctaaaatcttc
cctgagtattttgaactcctcttgacaatgtccccgcgtacctnggccgngaccacgcta
agggcgaattcttgcanaaatccatcacactggccggnccgntcnancatgcatntanag
ggcccaattcgcctanagggagtcgattacaatcnctggnccgncgttn

Table 5

Clone B39

Clone Sequence: (1-851) *(SEQ ID NO:5)*

gggnaattcggnatttaggnacggccgccagtgtgctggaattcgcccttagcgtggtcg
cggccgaggtacagccacaacttagaaaataaagcacacaagtattttggtgatttttac
aaatttttttttcaggtgtaaaggctacaaaaaattcctaaaaattagagaacactgaa
aacattaaaagtttnttctgactttatagtatttccattttaccctgaagacaacttaa
aaaatatgaccttcttagaacaggtcaccttgctataatattataaaaaattggtgagag
caagaaaaatgttcactggttatgcaggggtttgtaaatggtttctccccaaaccattag
gaaaaaaaaaaaagaagtacctgcccgggcggccgctcgaaagggcgaattctgcagata
tccatcacactggcggccgctcgagcatgcatctagagggcccaattcgccctatagtga
gtcgtattacaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggccg
ttacccaacttaatcgccttgcagcacatccccctttcgccagctggccgtaatagcgaa
gaggcccgcaccgatcgcccttcccaacagttgcgcagcctatacgtacggcagtttaag
gtttacacctataaaagagagagcccgttatccgnctgtttgggggatgtacagnagtga
tattattgacccccgggggccnccggatgggggacccccctgcccgtgcccgtttgctggc
nnanaaagnnccccntgaccttacccgggggggcctatcggggatnaaactggccctganac
ccccattggcc

Table 6

Clone Y68

Clone Sequence: (1-966) *(SEQ ID NO:6)*

tgggaccanctcgttttctnggnaaccccgccagtgtgctggaattcgccctttttttgc
ggccgcccgggccngtaccagagtttgcggacagcgatcctgccaacattgtgcacgact
tcaacaagaaactcactgcttatttggaccttaacctggacaagtactacgtgattcctc
tgaacacttccatcgttntnncgcccaaaaangngntggagctccttattaacattaagg
ccgggacctacctgcctcagtcctaccttatccatgagcacatggtgatcaccgaccgca
tcgagaacgtggacaacctgggcttcttcatctaccgactgtgtcacgacaaggagacct
acaaactgcagcgccgggaaacaattagaggtattcagaagcgggaagccagtacctgtt
tcaccattcggcattttgagaacaaatttgctgtggagactttaatttgttcttgagaag
tcaagaaaaaacgtggggaggaattcaatgccacagcataccctgccccttttgtattttg
tgcagtgattgtttttttaaaatcttcttttcatgtaagtagcaaacagggcttcactgtc
tcttcatctcaataactcaattaaaaaccattatcttaaaaaaagaaaacaaaacctttc
ttttttctaagtgtggtgctttgaagnttgaaatagcaaatgtgcagggtcctagataag
atcgnttctcnangagctacctactaggaanatctaaatggttggaaaacatancngaat
ttggggtaatttnnnctncatgaggaaaaacctaagaanancntncatnntaangaccca
nngnntgttgaanctnccccaccatngtncntgaggcnttcncnctnanaaggccgtcaa
nnntacntnantaactnntaacngctngnaattaaatganaacctncannnnttgngaaa
nacaag

Table 7

Clone Y91

Clone Sequence: (1-806) *(SEQ ID NO:7)*

tnnctcggttccctagtaacngccgccagtgtgctggattcgccctttcgaggcggccgc
ccgggcaggtacagnacacataaagnaggagtcaccagggnaagaagaataaaggcagat
tccaggttcagaaatatgcacagnatgctcccaagnaggcccaagnagcccgacactgag
agtccaaagtcttatgtgcatgtgggatcacagagatagtcaatgtagctgtctaagcca
actttggaacagccaatcagagcttgaatgacaggatgtatatagagatccatatgcaga
ctctgtcccagagccctggacagaatcatgagaacttggtgagcttcaggtctacttggg
gttattgactttggccataaagaggatactctgagcacttgtgtgatagataacaatcag
gaatggcctgttgaaacacacagctggtaatacggccttacgaatgccaccaataacccc
tgtggcagcagctgcttctgtgcctgtctcagccacatccagcacagccttgtggaccac
ctgagacacactcagtttcttggcttctgtgatcccagataggtcagcttgttcttgtga
agacttccttaatccccatttctggaaggacttnctctttccagcctgtagtccttagcc
attggagaacttgggccnggtttagcttcctntatttggctggaaaacaaagttttcctt
cattttctcggggctntggttgnaagntggncttccacctgctgntccttgccttggnca
gggaggattanncnggccttgctttc

Table 8

Clone Y92

Clone Sequence: (1-832) *(SEQ ID NO:8)*

cagagggganggaattcgcncctagcgtggtcgcggccgaggtacaacatcatctacaata
tgtcctctgatggacgtttgtcccaccatgcctgccagattgctcacgagcacncagatg
gagtgatcaagatgaggaagtctcagctgcagaatgaggaagagctgcagaaggccagga
agaagagacacttggatttcttggacatcctcttgtttgccagaatggaggataggaaca
gcttgtctgatgaggacctgcgtgcagaggtggacacattcatgtttgagggtcatgaca
ctacagccagtggaatttcctggattttctatgctctggccacccaccctgagcaccaac
agagatgcagagaggaggtgcagagcattctgggtgatggaacctctgtcacatgggacc
atctgggccagatgccctacaccaccatgtgcatcaaggaggccctgaggcactatccac
cagtaatatctgtgagtcgagagctcagctcacctgtcaccttcccagatggacgctcca
tacccaaaggtatcacagccacaatttccatttatggcctacatcataacccacgtttct
ggccaaaccaaaggtgtttgacccctctagatttgcaccagattcttctcaccataccat
gcttatctgccattctcaggaaggatcaangaactgcantgggaaancagtttgctatg
aacnaacttgaaggnggcttgtggnccttgaccctgctnccctttgaatgcttccanat
cccccaggatccaancccattgcaaganttgtgntgannncaaaaangnan

Table 9

Clone Y89

Clone Sequence: (1-842) *(SEQ ID NO:9)*

gntncagtnnnnttttnnnagtggattctccccagcgtggncncggccgaggtacctgcca
cctgcgagcccattcagagcgtctatttcttctctggagacaaatactaccgagttaacc
ttagaacccggcgagtggactctgtgaatcctccctacccacgctccattgctcagtatt
ggctgggctgcccgacctctgagaagtaggaatcagagcccactcggctgagcttcagga
gcctcatctctttctcccagcccaataaaaagtctgttggctacgaannnttaaaaaaa
aaaaagaggggaggaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaannttnttttttt
ttttnttnacccgcnnaaggggcncccnnccngggngncancntannngnggagnctnct
gcgaaaggnnccccnnaangnnggccnttaaaaggcntgnatttcnangcgacagngngn
ccccatacantgtcnctnctncnntctcgncgacccccctatanctngcaaaagcnctngg
ggggagaaaacncccnngaatctcccaaannataaaaccccctngaancccnattcnncn
tatntatanagaannngnccnttncncacnaaannnnccntttccaaaannggnngcttan
cataaaaanggccngcctcntnnanagggnngccccnccagagatancnccccccaaacna
nnangcccttcctancnnccatagccgagaataacannanccncctctnttcccccnnng
ggnctcnccncgntggggatcccccgggnactgaaaaaancccctcaaaaaaacctccgng
gg

Table 10

Clone A53

Clone Sequence: (1-800) *(SEQ ID NO:10)*

nggtcctagtcngcgcagagngtggattcgcccnagcgtggtcgcggccgaggtctttgg
caccaaatatcatttaaaaatccttcccaaacactaatgaagcaagcatgccaatattaa
tcacagttcgttatcccatattttataaccatcctaaaaagcatttgaactgacttattg
gccatttcaggtgttttacagtgtagatgttaggaaatcagcaggcgccactgagaaaca
gcagaatggtagaaccacacagaagaagccagaggtccggtctcacataagacgcttcgt
cctttcagaattaaaggactcatttcactaagcatcgcctccaaagcaagtcacagtcct
tcttgctatcaatgaacctggtaattttaccacatggtttctactctctgacagggccag
gatagaaatgagtcccaagcttcctaatcaaaaatttaaattttgacttttttccaaatca
tttaactggggatgaacagaccaaggcaggaaaagaaaacaaagttctagcaatcatttg
actcccagaaacactctgaactcagttgaactacaaccgatgtgaatgaacacctgctaa
acaggaaggtcaagaatgggggaccctcagttaatattgcacctcaatagaaagataacc
caaaagcaatattgatagtaagttctgccctggagattcactaaaatgccaaatcgnaaa
aaattaaaatttattacacaaatctcgctccttggggccaatctcaggacccaaagacag
gaactaactgncagnggact

Table 11

Clone Z19:

Clone Sequence: (1-953) *(SEQ ID NO:11)*

tttggggccactcggactntagtacggccgcagtgtgctggaattcgccctttcgagcgg
ccgcccgggcaggtacttttttttttttttttttttttatgattgtttctgaattttac
ttttctttaaaatgtctgaaacactcagaaatcaaatttgtataattatttcattattta
aggatttgacatcaagaaatattttaattacagaaattttatgggtggagccatcatggt
gcatttgtggaagtctaagaataatttcaggaactacttnttccttcaactatgttggt
gccaggattgaactcanatcctcatggncgttaacaagattctttatccttggagtcacc
tctntagcataatacttgattactacagnatgngagattaattagcaatttatatgccnc
atgccttaaatcaaaaacaagtacttttttccattcctgacaccagtntatgaggatgtta
aatatcctgcatgttcccacaaggattcaatanaatttttcaaaaaatttgncngacana
atgaaggaangccgcctgnattctaanncaaaaccaccgagngggattntcttcagctga
tgnngcaattctcataaanaattgccaaaganananncntntnaataaagccctttttga
cntggagaacacacgcccanccnattantttttttatnttttgctggggtatnnaaanacc
cagcacccactttccttcancttccatnncttggccctcccctgaaangtcanaaaacc
tgncgnanngancngttaggcntccnanaaggnccnncccnnanntntntgggnatggnc
ctnnntggtgnnaannaaccnnnaatttccatnnntnanttcgaantgnaaaannttanc
nnntnntnccnaannagatgttatcngntnntctccnnncncntggcatcnag

This clone contains inserted sequences from two different mouse mRNAs: 1) Partial sequence (345bp) of Mus musculus RIKEN cDNA 2810007J24 gene (2810007J24Rik), mRNA: 2) Partial sequence (178bp) of Mus musculus, cytochrome P450, 3a11

Table 12

Clone A104

Clone Sequence: (1-751) *(SEQ ID NO:12)*

ggattctcccnagtccggncgaggacgcgggggccagaagctgaaccctattcacagtga
aatcagtaatttagccggctttgaggtggaggtcataatcaatcctaccaatgctgacat
tgaccttaaagatgacctaggaaacacactggagaagaagggcggcaaggagtttgtaga
agctgttctggaactccggaaaaagaacgggcccttggaggtagctggagctgctattag
tgcaggccatggcctgcctgccaagtttntgatccactgtaatagncctgtctggggtgc
agacaaatgtgaagaacttctagaaaaganggtnaaaaactgcttggctctagctgatga
cagaaagctgaaatccatcgccttcccatccattggcagcggcaggaacgggttcccgaa
gcagacagcggcccagctcattctgaaggccatctccagctactttgtctccacgatgtc
ctcctccatcaaaactgtgtacgcgggtgnatgtgcatcattgcagcatgctgtccagct
gggtgctgctggatggattacgcaggcaacaccactgctgtcttcctcctgcccgatgat
ggggaagatgcagccatctggagcaacctctccaacaaggagctcatctctcagttctgc
ctnaacangcgccaaaagcgatgcccanatccatattcccanaactgtccattctctgga
aactnnaacttgaagaaccttgagcccccg

In the above Tables, "N" denotes "unknown".

Introduction to Master Tables

Master Table 1:

Col. 1: The internal designation for the clone. The sequences for the clones appear in tables 1-12.

Col. 2: There are three pieces of information here: (1) The database accession number for the mouse gene "corresponding" to the clone as determined by database searching, (2) in parentheses, the E value for the alignment of the clone sequence to the mouse gene. It is the expected number of matches with the same or better alignment score that would have occurred through chance. The lower the E value, the more statistically significant the alignment. (3) the database accession number for the mouse protein corresponding to the mouse gene above.

Col. 3. "U/F". "U" means an unfavorable differential pattern of expression, "F", a favorable one. "N" means no difference in expression. Three values are given, in order, control (C) to hyperinsulinemic (HI), control to diabetic (D), and HI to D. F is C>IR, C>D or HI>D. U is C<HI, C<D, or HI<D. N is C=HI, C=D or HI=D.

Col. 4: A human protein deemed to correspond to the clone, identified by database accession number and by name. Note that more than one human protein may be so identified. The human proteins are listed in order of correspondence to the clone, from most to least closely corresponding.

Col. 5: The E value for the alignment of the query sequence set forth in col. 6 to the human protein set forth in col. 4. There is one entry for each human protein in col. 4.

Col. 6: The method used to align the human protein of col. 4 to the query sequence, and the identity of the query sequence. If the query sequence is a clone sequence, the method will be BLASTX (DNA vs. protein). If the query sequence is the mouse gene of col. 2, the method will again be BLASTX. If the query sequence is the mouse protein of col. 2, the method will be BLASTP (protein vs. protein).

If only one method and query sequence is stated for a

particular mouse clone entry, then it applies to the identification of all of the human proteins listed as corresponding.

Col. 7. The database accession number of the corresponding human gene. There is one entry for each human protein in col. 4.

Master Table 2:

Col. 1: Mouse clone ID.

Col. 2: Corresponding mouse gene and protein.

Col. 3: U/F.

Col. 4: The classes and subclasses of human proteins deemed to correspond to the mouse clone. This is the result of extrapolation from the data of Master Table 1.

Master Table 1 is divided into three subtables on the basis of the Behavior" in col. 3. If a gene has at least one favorable behavior, and no unfavorable ones, it is put into Subtable 1A. In the opposite case, it is put into Subtable 1B. If it shows both favorable and unfavorable behavior, it belongs to Subtable 1C. Master Table 2 is analogously divided into subtables 2A, 2B and 2C.

Based on the related human proteins defined in Master Table 1, Master Table 2 generalizes, if possible, as to classes of human proteins which are expected to have similar behavior. For a given mouse gene, several human protein classes may be listed because of the diversity of the human proteins found to be related. In some cases, the stated human protein classes may be hierarchial, e.g., one may be a subset of another. In other cases, the stated classes may be non-overlapping but related. And in yet other cases, the stated classes may be non-overlapping and unrelated. Combinations of the above are also possible.

Master Table 1

Clone	Mouse Gene (E Value) Protein	U / F	Human Protein: Human Protein Name	E Value	Query Seq.	Human Gene
Subtable 1A: Favorable Human Proteins/Genes						
Z41	NM_007818	FFN	NP_000767: cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 3	2.00e-61	Blast X	NM_000776
	(e = 0.0)		AAA35747: cytochrome P450 nifedipine oxidase	6.00e-59	Z41	J04449
	NP_031844		NP_059488: cytochrome P450, subfamily IIIA, polypeptide 4; nifedipine oxidase;	3.00e-59		NM_017460
			P450-III, steroid inducible; glucocorticoid-inducible P450; cytochrome P450,			
			subfamily IIIA (niphedipine oxidase), polypeptide 3			
			NP_000768: cytochrome P450, subfamily IIIA, polypeptide 5	1.00e-59		NM_000777
			NP_000756: cytochrome P450, subfamily IIIA, polypeptide 7	1.00e-55		NM_000765
			NP_073731: cytochrome P450, family 3, subfamily A polypeptide 43 isoform 1	1.00e-54		NM_022820
			NP_476436: cytochrome P450, family 3, subfamily A,polypeptide 43 isoform 2	1.00e-54		NM_057095
			NP_476437: cytochrome P450 polypeptide 43; cytochrome P450, subfamily IIIA,	1.00e-54		NM_057096
			polypeptide 43			
Z74	NM_009280	FFF	AAG31034: SYT/SSX4 fusion protein	7.00e-86	Blast P	AF257500
	(e = 0.0)		AAF79937: Unknown protein	4.00e-85	NP_03 3306	AF230662
	NP_033306		AAM00188: SYT protein	8.00e-80		AF244972
			AAK21314: SYT variant 1	5.00e-79		AF343880
			NP_005628: Synovial sarcoma, translocated to X chromosome	2.00e-66		NM_005637
Z19	XM_133188	FFN	NP_003158: sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA) -preferring, member 1; sulfotransferase family 2A, dehydroepiandrosterone (DHEA) -preferring, member 1	2.00e-79	Blast X	NM_003167
	(e-173)		AAA35758: dehydroepiandrosterone sulfotransferase	2.00e-79	XM_13 3188	L20000
	XP_133188		CAA49755: dehydroepiandrosterone sulfotransferase	3.00e-79		X70222

			AAC51353: dehydroepiandrosterone sulfotransferase	3.00e-79		U13056
			AAH20755: Protein for MGC:22602	3.00e-79		BC020755
			AAB23169: alcohol/hydroxysteroid sulfotransferase	3.00e-79		S43859
			CAA59274: alcohol sulfotransferase; hydroxysteroid sulfotransferase	3.00e-79		X84816
			I38548: alcohol sulfotransferase	3.00e-79		I38548
			AAA75491: dehydroepiandrosterone sulfotransferase	3.00e-79		L36191
			AAA17750: dehydroepiandrosterone sulfotransferase	3.00e-79		U08025
			AAA17749: dehydroepiandrosterone sulfotransferase	3.00e-79		U08024
			Q06520: alcolol sulfotransferase; (DHEA-ST) (ST2) (ST2A3)	3.00e-79		L20000
			2021281A: dehydroepiandrosterone sulfotransferase	3.00e-79		2021281A
			AAC78553: hydroxysteroid sulfotransferase SULT2B1a	6.00e-56		U92316
			NP_004596: sulfotransferase family, cytosolic, 2B, member 1; sulfotransferase family 2B, member 1	6.00e-56		NM_004605
			AAC78498: hydroxysteroid sulfotransferase SULT2B1a	6.00e-56		U92314
			AAH34694: sulfotransferase family, cytosolic, 2B, member 1	6.00e-56		BC034694
			AAC78554: hydroxysteroid sulfotransferase SULT2B1b	6.00e-56		U92317
			AAC78499: hydroxysteroid sulfotransferase SULT2B1b	6.00e-56		U92315

Subtable 1B: Unfavorable Human Proteins/Genes

A104	XM_127380	UUN	NP_004884: H2A histone family, member Y isoform 2; histone macroH2A1.2; histone macroH2A1.1	2.00e-80	Blast X	NM_004893
	(e = 0.0)		AAC39908: histone macroH2A1.2	2.00e-80	A104	AF054174.
	XP_127380		NP_613258: H2A histone family, member Y isoform 3; histone macroH2A1.2; histone macroH2A1.1	9.00e-80		NM_138610
			H2AY_HUMAN: Core histone macro-H2A.1 (Histone macroH2A1) (mH2A1) (H2A.y) (H2A/y)	1.00e-79		AF041483
			NP_613075: H2A histone family, member Y isoform 1; histone macroH2A1.2; histone macroH2A1.1	1.00e-66		NM_138609
			AAH13331: Unknown (protein for MGC:13692)	2.00e-80		BC013331
B39	AY029764	UUN	AAH37738: Unknown (protein for MGC:33851)	0	Blast X	BC037738
	(e = -121)		NP_055109: translocating chain-associating membrane protein; translocating chain-ssociating membrane protein	0	AY029764	NM_014294
	AAK38167		AAH00687: translocating chain-associating membrane protein	0		BC000687
			CAA45218: TRAM protein	0		X63679
			S30034: translocating chain-associating membrane protein	0		
			Q15629: TRAM protein (Translocating chain-associating membrane protein)	0		X63679
			NP_689615: hypothetical protein MGC26568	e-135		NM_152402
			AAH30831: similar to TRAM protein (Translocating chain-associating membrane protein)	e-135		BC030831
			XP_068144: similar to TRAM protein	e-135		XM_068144
			BAC11091: unnamed protein product	e-135		AK074617
			AAH28121: TRAM-like protein	1.00e-99		BC028121
			NP_036420: TRAM-like protein; KIAA0057 gene product	1.00e-99		NM_012288
Y68	AK076139	UUN	NP_068839: integral membrane protein 2B	1.00e-65	Blast X	NM_021999
	(e = 0.0)		AAH16148: Similar to integral membrane protein 2B	1.00e-65	Y68	BC016148

	BAC36212		AAH00554: Similar to integral membrane protein 2B	1.00e-65		BC000554
	or:		AAD40370: putative transmembrane protein E3-16	1.00e-65		AF092128
	AB030203		AAG49434: putative transmembrane protein E3-16	1.00e-65		AF136973
	(e = 0.0)		AAD45280: transmembrane protein BRI	1.00e-65		AF152462
	BAA92766		Q9Y287: Integral membrane protein 2B	1.00e-65		AF152462
			AAF66130: transmembrane protein BRI	3.00e-65		AF246221
			BAA91210: unnamed protein product	5.00e-65		AK000503
Y89	BC018521	UUN	CAA28659: S-protein	3.00e-21	Blast X	X05006
	(e-128)		P04004: Vitronectin precursor (Serum spreading factor) (S-protein) (V75) [Contains: Vitronectin V65 subunit; Vitronectin V10 subunit; Somatomedin B]	3.00e-21	Y89	X03168
	AAH18521		NP_000629: vitronectin precursor; serum spreading factor; somatomedin B; complement S-protein	3.00e-21		NM_000638
			SGHU1V: vitronectin precursor [validated]	3.00e-21		
Y91	X56786	UUU	AAA51560: alpha-1-antichymotrypsin precursor	9.00e-12	Blast X	J05176
	(e = 0.0)		CAA25459: alpha 1 antichymotrypsin	9.00e-12	Y91	X00947
	CAA40106		CAA48671: alpha1-antichymotrypsin	9.00e-12		X68733
			AAH13189: Unknown (protein for IMAGE:3925035)	9.00e-12		BC013189
			1QMNA: Chain A, Alpha1-Antichymotrypsin Serpin In The Delta Conformation (Partial Loop Insertion)	9.00e-12		
			AAD08810: alpha-1-antichymotrypsin precursor	9.00e-12		AF089747
			AAH34554: serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	9.00e-12		BC034554
			P01011: Alpha-1-antichymotrypsin precursor (ACT)	9.00e-12		K01500

Subtable 1C: Mixed Human Genes/Proteins						
Y92	NM_007822	FNU	NP_000769: cytochrome P450, subfamily IVA, polypeptide 11; fatty acid omega-hydroxylase; P450HL-omega; alkane-1 monooxygenase; lauric acid omega-hydroxylase	3.00e-81	Blast X	NM_000778
	(e = 0.0)		Q02928: Cytochrome P450 4A11 precursor (CYPIVA11) (Fatty acid omega-hydroxylase) (P-450 HK omega) (Lauric acid omega-hydroxylase) (CYP4AII) (P450-HL-omega)	3.00e-81	Y92	L04751
	NP_031848		I65981: fatty acid omega-hydroxylase (EC 1.14.15.-) cytochrome P450 4A11 - human	3.00e-81		gi:2117372
			BAA02864.1: fatty acid omega-hydroxylase	9.00e-79		D13705
			AAF76722.1: fatty acid omega-hydroxylase CYP4A11	1.00e-80		AF208532
			CAB72105.1: dJ18D14.4 (cytochrome P450, subfamily IVA, polypeptide 11)	4.00e-78		AL135960
			O4HUB1: cytochrome P450 4B1 - human	2.00e-59		GI:65678
			AAL57720.1: cytochrome P450	2.00e-59		AY064485
			AAM09532.1: cytochrome P450	2.00e-59		AF491285
			NP_000770.1: cytochrome P450, subfamily IVB, polypeptide 1; cytochrome P450, subfamily IVB, member 1; microsomal monooxygenase	2.00e-59		NM_000779
			AAL57721.1: cytochrome P450	2.00e-59		AY064486
			BAA75823.1 : Leukotriene B4 omega-hydroxylase	4.00e-53		AB015295
			NP_001073.3 : Cytochrome P450, subfamily IVF, polypeptide 2; leukotriene B4 omega-hydroxylase; leukotriene-B4 20-monooxygenase	5.00e-53		NM_001082
			NP_000887.1 : cytochrome P450, subfamily IVF, polypeptide 3; leukotriene B4 omega hydroxylase; leukotriene-B4 20-monooxygenase; cytochrome P450-LTB-omega	1.00e-53		NM_000896
			AAC50052.2: cytochrome P450 4F2	6.00e-52		U02388
			Q9HBI6 : Cytochrome P450 4F11 (CYPIVF11)	7.00e-53		AF236085.
			NP_067010.1 : cytochrome P450, subfamily IVF, polypeptide 11	> e-50		NM_021187
			Q9HCS2 : Cytochrome P450 4F12 (CYPIVF12)	7.00e-53		AY008841
			NP_076433.1 : cytochrome P450 isoform 4F12	7.00e-53		NM_023944
			AAH35350.1 : similar to cytochrome P450	7.00e-53		BC035350
			NP_009184.1 :cytochrome P450, subfamily IVF, polypeptide 8; microsomal monooxygenase; flavoprotein-linked monooxygenase	> e-50		NM_007253
			XP_065069.2 : similar to CYTOCHROME P450 4F6 (CYPIVF6)	> e-50		XM_065069
			XP_029070.2 : similar to Cytochrome P450 4F12 (CYPIVF12)	> e-50		XM_029070
			AAH22851.1 : Similar to cytochrome P450, subfamily IVA, polypeptide 11	> e-50		BC022851
			XP_065068.1 : similar to Cytochrome P450 4F12 (CYPIVF12)	> e-50		XM_065068

A17	AF453874	UUF	NP_006206:serine (or cysteine) proteinase inhibitor, clade A	1.00e-24	Blast X	NM_006215
	(e = 0.0)		(alpha-1 antiproteinase, antitrypsin), member4; protease ingibitor 4 (kallistatin)		AF453874	
	AAL51002		CAD66567: unnamed protein product	1.00e-24		BX248760
			AAH14992: Unknown (protein for MGC:23251)	1.00e-24		BC014992
			CAD62337: unnamed protein product	1.00e-24		BX248009
			AAC41706: kallistatin	1.00e-24		L28101
			AAA59454: kallistatin	1.00e-24		L19684
			P29622: Kallistatin precursor (Kallikrein inhibitor) (Protease inhibitor 4).	1.00e-24		L19684
A53	AK050237	UNF	NP_004489: one cut domain, family member 1; hepatocyte nuclear factor 6, alpha	5.00e-42	Blast X	NM_004498
	(e = 0.0)		AAB61705: hepatocyte nuclear factor 6	9.00e-42	AK050237	U77975
			NP_004843: one cut domain, family member 2; onecut 2	3.00e-33		NM_004852
B8	AY152393	UNF	NP_775491: liver-specific uridine phosphorylase	7.00e-21	Blast X	NM_173355
	(e =0.0)		XP_087230: similar to Uridine phosphorylase (UDRPase)	7.00e-21	B8	XM_087230
	AAO05705		AAH33529: Similar to uridine phosphorylase	7.00e-21		BC033529
			AAD12227: similar to uridine phosphorylase; similar to Q16831 (PID:g2494059)	7.00e-21		AC005539

Master Table 2

Clone	Mouse Gene / Protein	U / F	Human Protein Class
Subtable 2A: Favorable Human Protein Classes			
Z41	NM_007818	FFN	cytochrome P450
	NP_031844		Subclass: Cytochrome P450 3A4 (Quinine 3-monooxygenase) (CYPIIIA4) (Nifedipine oxidase) (NF-25) (P450-PCN1)
			Subclass: cytochrome P450, subfamily IIIA, polypeptide 4; nifedipine oxidase; P450-III, steroid inducible; glucocorticoid-inducible P450; cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 3
			Subclass: cytochrome P450, subfamily IIIA, polypeptide 5; niphedipine oxidase; aryl hydrocarbon hydroxylase; xenobiotic
			Subclass: cytochrome P450, subfamily IIIA, polypeptide 7; aryl hydrocarbon hydroxylase; microsomal monooxygenase; xenobiotic monooxygenase; flavoprotein-linked monooxygenase
			Subclass: cytochrome P450 polypeptide 43 isoform 2; cytochrome P450 polypeptide 43
			Subclass: cytochrome P450 polypeptide 43 isoform 1; cytochrome P450 polypeptide 43
			Subclass: cytochrome P450 polypeptide 43 isoform 3; cytochrome P450 polypeptide 43
Z74	NM_009280	FFF	SYT/SSX4 fusion protein
	NP_033306		Alternate: SYT protein
			Alternate: SYT variant 1
			Alternate: Synovial sarcoma, translocated to X chromosome
Z19	XM_133188	FFN	Sulfotransferase
	XP_133188		Subclass: alcohol sulfotransferase; hydroxysteroid sulfotransferase
			Subclass: alcolol sulfotransferase; (DHEA-ST) (ST2) (ST2A3)
			Subclass: dehydroepiandrosterone sulfotransferase
			Subclass: sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)
			-preferring, member 1; sulfotransferase family 2A,
			dehydroepiandrosterone (DHEA) -preferring, member 1
			Subclass: sulfotransferase family, cytosolic, 2B, member 1
			Subclass: hydroxysteroid sulfotransferase SULT2B1a
			Subclass: hydroxysteroid sulfotransferase SULT2B1b

Subtable 2B: Unfavorable Human Protein Classes			
A104	XM_127380	UUN	Histone
	XP_127380		Subclass: H2A histone family, member Y isoform 2; histone macroH2A1.2; histone macroH2A1.1 (mH2A1)
B39	AY029764	UUN	TRAM protein (Translocating chain-associating membrane protein)
	AAK38167		
Y68	AK076139	UUN	Intergal Membrane Protein
	BAC36212		Subclass: integral membrane protein 2B
	OR		Subclass: putative transmembrane protein E3-16
	AB030203		Subclass: transmembrane protein BRI
	BAA92766		
Y89	BC018521	UUN	Vitronectin
	AAH18521		Subclass: Vitronectin precursor (Serum spreading factor) (S-protein) (V75) [Contains: Vitronectin V65 subunit; Vitronectin V10 subunit; Somatomedin B]
Y91	X56786	UUU	Protease
	CAA40106		Subclass: alpha 1 antichymotrypsin
			Subclass: serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3

Subtable 2C: Mixed Human Protein Classes			
Y92	NM_007822	FNU	cytochrome P450,
	NP_031848		Subclass: cytochrome P450, subfamily IVA, polypeptide 11; fatty acid omega-hydroxylase; P450HL-omega; alkane-1 monooxygenase; lauric acid omega-hydroxylase (CYP4A11)
			Subclass: cytochrome P450, subfamily IVB, polypeptide 1; cytochrome P450, subfamily IVB, member 1; microsomal monooxygenase
			Subclass: cytochrome P450, subfamily IVF, polypeptide 2; leukotriene B4 omega-hydroxylase; leukotriene-B4 20-monooxygenase
			Subclass: cytochrome P450, subfamily IVF, polypeptide 3; leukotriene B4 omega hydroxylase; leukotriene-B4 20-monooxygenase; cytochrome P450-LTB-omega
			Subclass: cytochrome P450, subfamily IVF, polypeptide 11
			Subclass: Cytochrome P450 4F12 (CYPIVF12)
			Subclass: cytochrome P450, subfamily IVF, polypeptide 8; microsomal monooxygenase; flavoprotein-linked monooxygenase
			Subclass: cytochrome P-450LTBV
A17	AF453874	UUF	Kallistatin
	AAL51002		Subclass: Serine (or cysteine) proteinase inhibitor, clade A
			(alpha-1 antiproteinase, antitrypsin), member4; protease ingibitor 4 (kallistatin)
			Subclass: Kallistatin precursor (Kallikrein inhibitor) (Protease inhibitor 4).
A53	AK050237	UNF	hepatocyte nuclear factor
			Subclass: one cut domain, family member 1; hepatocyte nuclear factor 6, alpha
			Subclass: one cut domain, family member 2; onecut 2
B8	AY152393	UNF	Liver-specific uridine phosphorylase
	AAO05705		

CLAIMS

I/We hereby claim:

1. A method of screening for human subjects who are prone to progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, which comprises assaying tissue or body fluid samples from said subjects to determine the level of expression of

(I) at least one "favorable" human marker gene, said human marker gene encoding a human protein which is substantially structurally identical and/or conservatively identical in sequence to a reference protein which is (a) selected from the group consisting of mouse and human proteins set forth in master table 1, subtables 1A and 1C, or (b) selected from the group consisting of human proteins within at least one of the human protein classes set forth in master table 2, subtables 2A and 2C,

and directly correlating the level of expression of said marker gene with the propensity to progression in said patient,

(II) at least one "unfavorable" human marker gene, said human marker gene encoding a human protein which is substantially structurally identical and/or conservatively identical in sequence to a reference protein which is (a) selected from the group consisting of mouse and human proteins set forth in master table 1, subtable 1B and 1C, or (b) selected from the group consisting of human proteins belonging to at least one of the human protein classes set forth in master table 2, subtables 2B and 2C,

and inversely correlating the level of expression of said marker gene with the propensity to progression in said patient.

2. The method of claim 1 in which the human marker gene is a wholly favorable or a wholly unfavorable human marker gene.

3. The method of claim 1 in which the human marker gene is a favorable marker gene.

4. The method of claim 1 in which the human marker gene is an unfavorable marker gene.

5. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Z41.

6. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Z74.

7. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Y92.

8. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Z19.

9. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone A17.

10. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone A53.

11. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone A104.

12. The method of claim 1 in which the reference protein is

a human protein listed in master table 1 as corresponding to clone B8.

13. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone B39.

14. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Y68.

15. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Y89.

16. The method of claim 1 in which the reference protein is a human protein listed in master table 1 as corresponding to clone Y91.

17. The method of any one of claims 1-4 in which the reference protein is listed in master table 1.

18. The method of any one of claims 1-17 in which the level of expression of the marker protein is ascertained by measuring the level of the corresponding messenger RNA.

19. The method of any one of claims 1-17 in which the level of expression is ascertained by measuring the level of a protein encoded by said marker gene.

20. A method of protecting a human subject from progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, which comprises administering to the subject a protective amount of at least one agent which is

(I)

(1) a polypeptide which is substantially structurally

identical and/or conservatively identical in sequence to a reference protein which is (a) selected from the group consisting of mouse and human proteins set forth in master table 1, subtables 1A and 1C, or (b) selected from the group consisting of human proteins within at least one of the human protein classes set forth in master table 2, subtables 2A and 2C, or

(2) an expression vector encoding the polypeptide of (I)(1) above and expressible in a human cell, under conditions conducive to expression of the polypeptide of (I)(1); or

(II)

(1) an antagonist of a polypeptide, occurring in said subject, which is substantially structurally identical and/or conservatively identical in sequence to a reference protein which is (a) selected from the group consisting of mouse and human proteins set forth in master table 1, subtable 1B and 1C, or (b) selected from the group consisting of human proteins belonging to at least one of the human protein classes set forth in master table 2, subtables 2B and 2C,

(2) an anti-sense vector which inhibits expression of said polypeptide identified in (II)(1) above in said subject,

where said agent protects said subject from progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state.

21. The method of claim 20 in which the reference protein is set forth in master table 1, subtable 1A or 1B, or is of a human protein class set forth in master table 2, subtable 2A or 2B.

22. The method of claim 20 in which (I) applies.

23. The method of claim 20 in which (II) applies.

24. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone Z41.

25. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone Z74.

26. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone Y92.

27. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone Z19.

28. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone A17.

29. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone A53.

30. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone A104.

31. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone B8.

32. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone B39.

33. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone Y68.

34. The method of claim 20 in which the reference protein is listed in master table 1 as corresponding to clone Y89.

35. The method of claim 20 in which the reference protein is

listed in master table 1 as corresponding to clone Y91.

36. The method of any one of claims 1-4, 18-23 in which the reference protein is listed in master table 1.

37. The method of any one of claims 1-4, 18-23, 37 in which the reference protein is a human protein.

38. The method of any one of claims 1-4, 18-23, 37 in which the reference protein is a mouse protein.

39. The method of any one of claims 1-38 in which said polypeptide is substantially structurally identical to said reference protein.

40. The method of any one of claims 1-38 in which said polypeptide is at least 80% identical to said reference protein.

41. The method of any one of claims 1-38 in which said polypeptide is at least 90% identical to said reference protein.

42. The method of any one of claims 1-41 in which said polypeptide is at least conservatively identical to said reference protein.

43. The method of any one of claims 1-41 in which said polypeptide is at least highly conservatively identical to said reference protein.

44. The method of any one of claims 1-38 in which said polypeptide is identical to said reference protein.

45. The method of any one of claims 1-44 in which the E-value cited for the reference protein in Master Table 1 is not more than e-20.

46. The method of claim 45 in which the E-value cited for the reference protein in Master Table 1 is less than e-40, more preferably less than e-50, even more preferably less than e-60, considerably more preferably less than e-80, and most preferably less than e-100.

47. The method of any one of claims 1-46 in which the agent is a DNA, or is a polypeptide encodable by a DNA, which specifically hybridizes to the recited DNA strand of any of SEQ ID NOs: 1-12, or to the complementary strand thereof.

48. A method of screening for human subjects who are prone to progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, which comprises

assaying tissue or body fluid samples from said subjects to determine the level of expression of at least one human marker protein, where said human marker protein is identifiable as a homologue of a mouse marker gene which is expressed at different levels in a first group of mice who are experiencing or are prone to such progression and in a second group of mice protected against or otherwise less prone to such progression, and

correlating said level of expression of said human marker gene with the propensity to such progression in said subject.

49. The method of claim 48 in which the first group of mice belong to a mouse model of type II diabetes and the second group of mice are non-diabetic mice with normal insulin resistance.

50. The method of claim 48 in which the first group of mice belong to a mouse model of type II diabetes and the second group of mice belong to a non-diabetic mouse model of hyperinsulinemia.

51. The method of claim 48 in which the first group of mice

belong to a non-diabetic mouse model of hyperinsulinemia and the second group of mice are non-diabetic mice with normal insulin levels.

52. The method of any one of claims 48-51 in which the marker gene is one expressed more strongly in the first group of mice than in the second group of mice.

53. The method of any one of claims 48-51 in which the marker gene is one expressed more strongly in the second group of mice than in the first group of mice.

54. The method of any one of claims 48-51 in which said reference protein is identifiable as a homologue by a BLASTN or BLASTX search conducted, using any of SEQ ID NOS:1-12 as a query sequence, on the NCBI Entrez sequence database(s), on or before the filing date of the instant application, and the E value calculated by BLASTX or BLASTN for the alignment of that homologue, or cDNA encoding that homologue, to the query sequence is less than e-10.

55. The method of claim 54 in which the E value calculated by BLASTN or BLASTX would be less than e-15, more preferably less than e-20, still more preferably less than e-40, further more preferably less than e-50, even more preferably less than e-60, considerably more preferably less than e-80, and most preferably less than e-100.

56. A method of protecting a human subject from progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, which comprises administering to the subject a protective amount of at least one agent which

(1)

(a) down-regulates expression of an "unfavorable" protein which is identifiable as a homologue in said subject

of a mouse marker gene which is

(i) up-regulated in a first group of mice, which are experiencing or are prone to progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, and/or

(ii) down-regulated in a second group of mice, which are protected against or otherwise less prone to progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state, relative to the other group, or

(b) is an antagonist for the expression product of the "unfavorable" gene, or

(c) degrades that product,

or

(2)

(a) up-regulates expression of a "favorable" protein which is identifiable as a homologue in said subject of a mouse marker gene which is

(i) down-regulated in said first group of mice and/or

(ii) up-regulated in said second group of mice, relative to the other group or

(b) is an agonist for the favorable protein, or

(c) inhibits the degradation of the favorable protein, or

(d) is said favorable protein or a protein which is substantially or conservatively identical thereto, or

(e) is an expression vector comprising a DNA sequence encoding said protein (d) and operably linked to a promoter whereby said protein (d) is expressed in cells of said subject which are transformed by said vector,

where said agent protects said subject from progression from a non-diabetic normoinsulinemic state to a non-diabetic hyperinsulinemic state, or from either to a type II diabetic state.

57. The method of any one of claims 1-56 in which the human subject is overweight.

58. The method of any one of claims 1-56 in which the human subject is obese.

59. The method of any one of claims 1-58 in which the human subject is at least age 45.

60. The method of any one of claims 1-59 which further comprises determining the blood pressure, HDL cholesterol level or triglyceride level of the subject.

61. The method of any one of claims 1-60 in which the human subject is hypertensive.

62. The method of any one of claims 1-61 in which the human subject has an HDL cholesterol level of more than 35 mg/dL and/or a triglyceride level of at least 250 mg/dL.

63. The method of any one of claims 1-61 in which the subject is a human diabetic and also has an fasting plasma insulin level of more than 26 micro-IU/ml.

64. The method of any one of claims 1-61 in which the subject is a human diabetic and also has an fasting plasma insulin level of not more than 26 micro-IU/ml.